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The analysis of free fatty acids in biological fluids HPLC

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THE ANALYSIS OF FREE FATTY ACIDS IN BIOLOGICAL FLUIDS BY HPLC.

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SUMMARY

Techniques for derivatisation of fatty acids to form compounds suitable for detection by High Performance Liquid Chromatography using a 254 nm. ultraviolet detector have been developed.

Benzylation of the fatty acids by a mild, thermal decomposition of the benzyldimethylanilinium salts of the fatty acids has been shown to proceed essentially to completion. Separation of the benzyl esters of the naturally occurring fatty acids in human physiological fluids was accomplished using a methanol/water elution from a reverse phase column.

Quantitative formation of the 4-nitrobenzyl esters of the same acids, resulting in enhanced ultraviolet absorption with a concomittant increase in sensitivity in HPLC separations, was developed. Once again separations were accomplished on the reverse phase column, a methanol/water gradient as eluent.

The increased sensitivity allowed application of the 4-nitrobenzyl derivatisation to the analyses of free fatty acids in microsamples of physiological fluids. Acids from C12:0 to C18:3 were extracted and quantified, using n-eicosanoic acid as internal standard, from whole blood and plasma. Levels of the acids were in the normal range. In addition, a blood sample spiked with phytanic acid, the

presence of which could indicate that the donor suffers from Refsum's Syndrome, was successfully analysed.

Extraction and quantitation of free fatty acids in microsamples of dried blood spots was also accomplished by this method.

CONTENTS.

I N T R O D U C T I O N

Recently, there has been interest in developing high performance liquid chromatographic (HPLC) methods for the determination of long chain fatty acids. Durst et al[1] have thoroughly reviewed these developments and pointed out the need to derivatise these acids in order to enhance their detectability by ultraviolet absorption detection systems.

(i) The Significance of Free Fatty Acids in Physiological Fluids.

Fatty acids are major fuels for animals, both as a primary supply from the diet and as a secondary supply created from other dietary components (e.g. from glucose - adipose tissue consumes 27 carbon atoms as glucose to store 16 carbon atoms as palmitate, with the remaining 11 appearing as CO_2[2]). They are stored and later released through the bloodstream to meet the demands of many tissues, especially muscles. Thus, plasma levels of total free fatty acids have been measured as indicators of fat mobilisation or physiological normalcy of the animal.

The oxidation of fatty acids occurs in the mitochondria, but the compounds are present in the cell outside of the mitochondria, either as free fatty acids from the blood, or as triglycerides that are hydrolysed in the cytosol.

Free fatty acids are transported in the blood, mainly in combination with serum albumin. About two milligrams of fatty acid are carried per gram of albumin[2], albeit that the turnover is rapid. Albumin is present in human blood plasma to levels of about 4 to 7 grams of protein per 100 millilitres[3], giving an indication that the total plasma free fatty acid content would be approximately 20 milligrams per 100 millilitres of plasma (20 mg.%) at any given instant.

This is verified by literature[4] wherein the non-esterified fatty acids in human plasma are listed as 8 - 20 mg.%. Harper[5] points out that total free fatty acids varies with the nutritional state, but ranges from 6 to 20 mg.%.

Fatty acids from food are converted into triglycerides by the intestinal mucosa which then forms the triglycerides into an emulsion (stabilised by the presence of a small quantity of protein).[2] The emulsion droplets (chylomicrons) may supply several tissues - the liver removes some, but for a well fed animal, the adipose tissue takes up the bulk. Free fatty acids arise in plasma from lipolysis of the triglycerides in adipose tissue, or as a result of the action of lipoprotein lipase during uptake of plasma triglycerides into tissues.[5]

The ratios of various free fatty acids in the blood would be expected to be approximately the same as those stored as triglycerides in the adipose tissue. It has been

TABLE 1

COMPOSITION OF TRIGLYCERIDES IN PLANT OILS AND IN ADIPOSE TISSUE OF HUMANS EATING THE OILS [6.]

PERCENTAGE OF TOTAL FATTY ACID RESIDUES - ADIPOSE TISSUE

Fatty Acid	Humans on Random American Diet	Corn Oil	Humans with 40% of Energy Source as Corn Oil for 3 years	Linseed Oil	Humans Eating 83 gm. of Linseed Oil Daily for 1 Year	Coconut Oil	Humans Eating 60 gm. of Coconut Oil for 1.5 years
8 : 0						8	
10 : 0						10	
12 : 0	0.7		0.1		0.1	44	14.5
14 : 0	3.3		0.7		1.2	13	13.9
16 : 0	19.5	12	15.3	6	14.7	6	17.5
16 : 1	6.9		2.2		5.8		7.6
18 : 0	4.2	2	2.2	4	5.4	2	2.7
18 : 1	46.3	29	32.1	22	35.5	14	30.4
18 : 2	11.4	58	45.2	15	20.5	2	9.3
18 : 3	0.4			52	13.7		0.1

Data for adipose tissue from J. Hirsch in "Handbook of Physiology", p. 148, section 5, American Physiological Society (1965).

shown [6] that ratios of the various fatty acid residues as triglycerides in the adipose tissue of humans is variable, within limits, dependent on dietary factors - particularly for unsaturated fatty acids (Table 1). Shorter chain length fatty acids (C8:0, C10:0) appear to have been modified (possibly by chain lengthening in the liver[2]) or have been disposed of elsewhere.

The levels shown correlate well with the figures for the saturated free fatty acids in blood spots determined by Mee, Korth and Halpern[7] which in turn were cross-referenced with other literature.[4,8]

Although the common fatty acids range up to C24:0, no figures are readily available as to levels of free fatty acids in human blood with numbers of carbons per molecule greater than 18. It is assumed any larger fatty acid molecules greater than C18 are metabolised to produce fatty acids with 16 or 18 carbons in the chain in normal circumstances.[2] However at least one disorder occurs in the metabolism of a larger fatty acid in some humans - known as the Phytanic Acid Storage Disease, or Refsum's Syndrome.

Refsum's syndrome is a rare inherited disorder of lipid metabolism characterised clinically by peripheral neuropathy (absent or diminished deep tendon reflexes), ataxia (disco-ordination and unsteady gait), retinis pigmentosa (failing night vision) and changes in the skin and bones.

This disorder is accompanied by accumulation in the tissues, especially in the liver and kidney, of phytanic acid, a 20 carbon branched chain acid (3,7,11,15-tetramethylhexadecanoic acid). The acid may comprise as much as 30% of the total fatty acids of the blood plasma of a patient suffering from Refsum's Syndrome[9], but only traces of this component are found in normal plasma and are generally undetectable in routine analyses.

The origin of the phytanic acid in these patients is proposed to be :

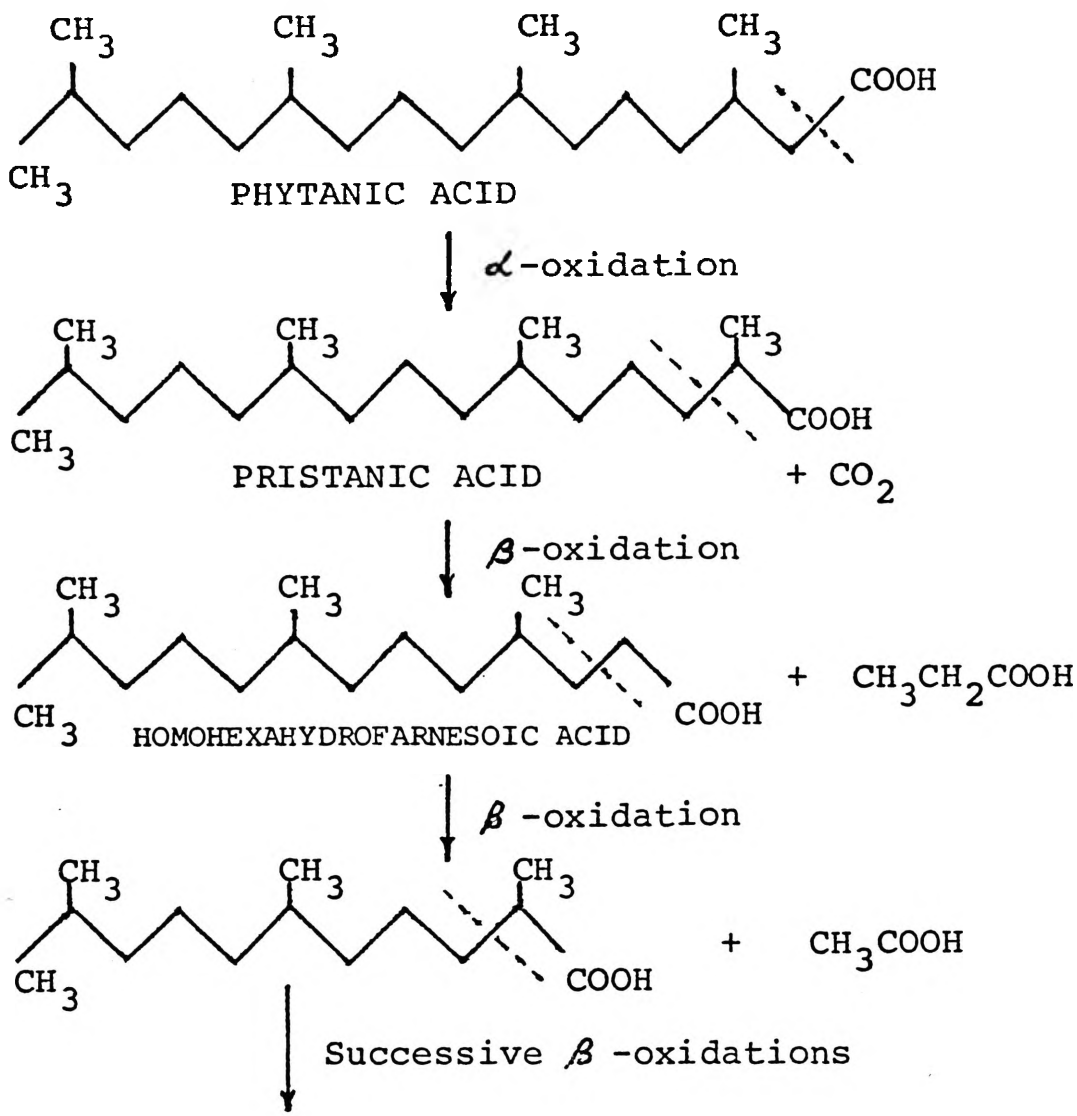
1. Dietary phytanic acid (butter[10], plasma from ruminants such as ox[11] and cow[12], and sheep fat[13])

and 2. Phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) - a component of the chlorophyll molecule. The phytol is readily converted to the phytanic acid, probably through the phytenic acid (3,7,11,15-tetramethylolec-2-enoic acid) intermediate.[14] Phytol is efficiently metabolised by normal human subjects (60 - 94% of an orally administered tracer dose) but similar results were obtained for two patients with Refsum's Syndrome.[15] However, studies of phytol adsorption in rats showed that only about 10 - 20% of the dose was converted to phytanic acid.

Studies by Baxter and Steinberg[16,17] showed not more than 2% of the bound chlorophyll phytol was absorbed and as such is apparently much less important than ingestion of preformed phytanic acid.

FIGURE 1

PROPOSED SCHEME FOR PHYTANIC ACID OXIDATION IN MAMMALIAN SYSTEMS.



PRODUCTS OF COMPLETE OXIDATION.

$$1\ CO_2 + 3\ CH_3CH_2COOH + 3\ CH_3COOH + 1\ \begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH\text{-}COOH$$

There are reasons to suspect that there may be other precursors of phytanic acid. Steinberg[18] points out that there is a close correlation of the total phytanic acid content of the tissues of several patients with their expected intake over a period of years. But patients do seem to have a limited capacity to metabolise and eliminate phytanic acid - rigid dietary control reduced the phytanic acid level. It was then suggested that additional sources of phytanate be tested - for example, endogenous biosynthesis by intestinal flora.[18]

Normal animals including man, appear to have a large capacity to dissimulate phytanic acid and prevent its accumulation, even at high levels of intake.[9] The rate of disappearance of phytanic acid from plasma is lower than that for palmitic acid (hexadecanoic acid, C16:0), however even exceptionally high levels of phytanic acid disappear over several weeks. This was determined by dosing rats with 2 - 5% by weight of phytol or phytanic acid in their diets until their liver and serum levels of phytanate were comparable to those of a patient, then monitoring the rate of removal.[20,21] (The point was made that extrapolation of these results to humans may not be justified.)

It is proposed that phytanic acid is oxidised through an initial α-oxidation to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and then further by β-oxidation steps. (Figure 1).

For patients suffering Refsum's Syndrome, the primary defect is failure to convert phytanic acid to α-hydroxyphytanic acid, the initial step in the conversion to pristinic acid.[22,23,24,25] This step is catalysed by phytanic acid α-hydroxylase, and activity of this enzyme is absent in the disease. To verify this, it was found that patients suffering from the disease did not accumulate pristinic acid when it was introduced into their diet; so presumably the metabolic pathway was not blocked to the β-oxidations in the proposed degradation (the second and subsequent steps in the proposed scheme for phytanic acid degradation in mammalian systems).

Consequently an analytical procedure to detect and monitor the levels of phytanic acid in human blood plasma is necessary for the diagnosis for people suffering from Refsum's Syndrome.

This project revolves around the distribution and analysis of the normal free fatty acids that occur naturally in human physiological fluids, as well as establishing a method of analysis for phytanic acid in the fluids.

Plasma levels of total free fatty acids were originally performed by extraction-titration methods.[41-44,31]

It was found that these techniques are subject to interferences by other titrateable substances which are also extracted from the sample.

A rapid and quantitative blood analysis for free fatty acids by chemical ionisation mass spectrometry has been reported recently by Mee, Korth and Halpern[6], but gas chromatography has been used for some time to determine the free fatty acid composition of tissue and sera.[45,46] Formation of the volatile methyl esters for gas chromatographic separation and quantitation has been the usual method, although analysis of the trimethyl silyl esters has also been reported.[47]

(ii) Chromatography.

High Performance Liquid Chromatography offers many advantages in natural product separation and analysis relative to gas chromatography. Chief among these is the potential to separate thermolabile and/or high molecular weight substances of low volatility at ambient temperatures.

The outstanding problem is to find a reasonably stable universal detector that will give a linear response over a wide concentration range of the analyte. The most stable detector commercially available, that has a useful sensitivity, is the ultraviolet absorption system utilising a flow through microcell (approximately 10 ul in size). Greater stability is obtained using a fixed wavelength type. (The most common is a low pressure mercury lamp source, utilising the 254 nm. or 280 nm. lines of the Hg emission spectrum.)

However use of the UV absorption system is not feasible unless the components of the sample mixture possess reasonably high extinction coefficients at the wavelength of interest. Unfortunately, naturally occurring fatty acids have low absorptivities, the saturated members having absorption maximums in the range 200 to 210 nms., with low extinction coefficients (less than 100).

e.g., Acetic acid; λ_{max} = 208 nm.

ε_{max} = 32 $l.mole^{-1}cm.^{-1}$

Additions of methyl groups to the chain have little effect on the UV absorptivities. (The presence of points of unsaturation in the fatty acids increase their absorptivities at about 200 nm. due to the double bonds.)

The saturated fatty acids palmitic (hexadecanoic) and stearic (octadecanoic) give no observable response using an ultraviolet detection system at 254 nm. using reverse phase column chromatography. It is customary to prepare the methyl esters of these fatty acids for gas chromatographic analyses, but these esters produce only a minimal response on a 254 nm. UV detector upon L.C. analysis. In contrast, the corresponding benzyl esters show significantly greater sensitivity as a result of the enhanced chromophoric properties of the benzenoid ring at 254 nm.

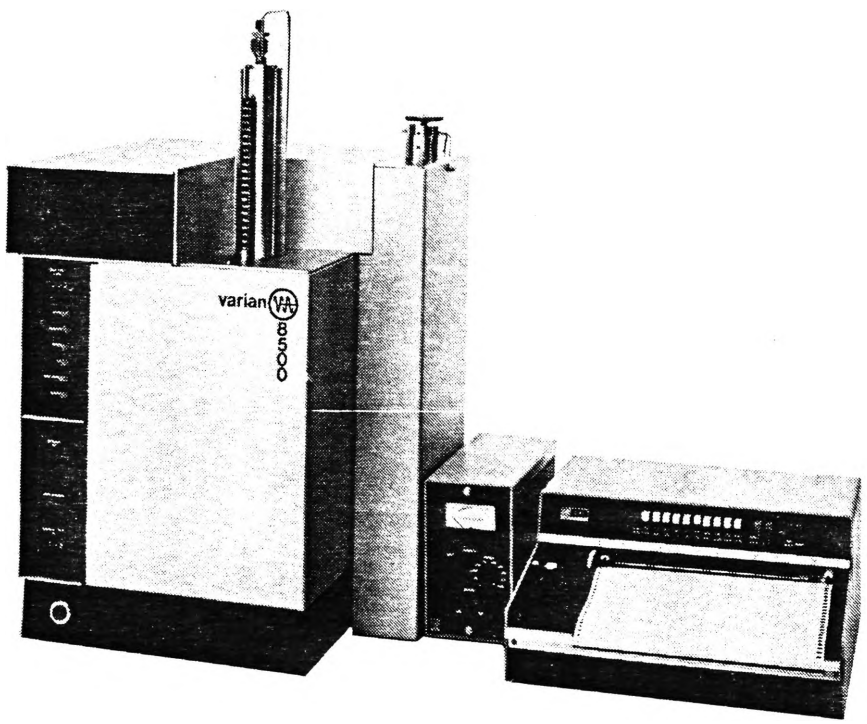
Separation of the fatty acids or their corresponding esters are best facilitated by using reverse phase column chromatography. All other things being equal, retention times of the components of a mixture of saturated fatty acids or

their esters in a reverse phase system will then be determined by the chain length of the fatty acid; the longer the non-polar chain, the longer the retention on the stationary reverse phase when employing polar eluents. (Similar separations on polar columns using non-polar eluents would be more difficult as the polarities of the long chain fatty acids, or corresponding esters, are very similar and thus their distribution coefficients under such conditions would be very close.) The retention times of unsaturated fatty acids, or corresponding esters, are dependant on the chain length, and the number of points of unsaturation.

Analysis of fatty acids by HPLC using a 254 nm. UV detector necessitates derivatisation of the acids. Mild, fast procedures for the formation of the benzyl and 4-nitrobenzyl esters of the naturally occurring fatty acids, and their quantitation by HPLC have been studied.

FIGURE 2

VARIAN AEROGRAPH MODEL 8500 HIGH PERFORMANCE LIQUID CHROMATOGRAPH.



E X P E R I M E N T A L

(i) Instrumentation.

All isocratic elutions were performed on a Varian Aerograph Model 8500 High Performance Liquid Chromatograph fitted with a Micropak CH10 column and a monochromatic UV detection system. (Figure 2).

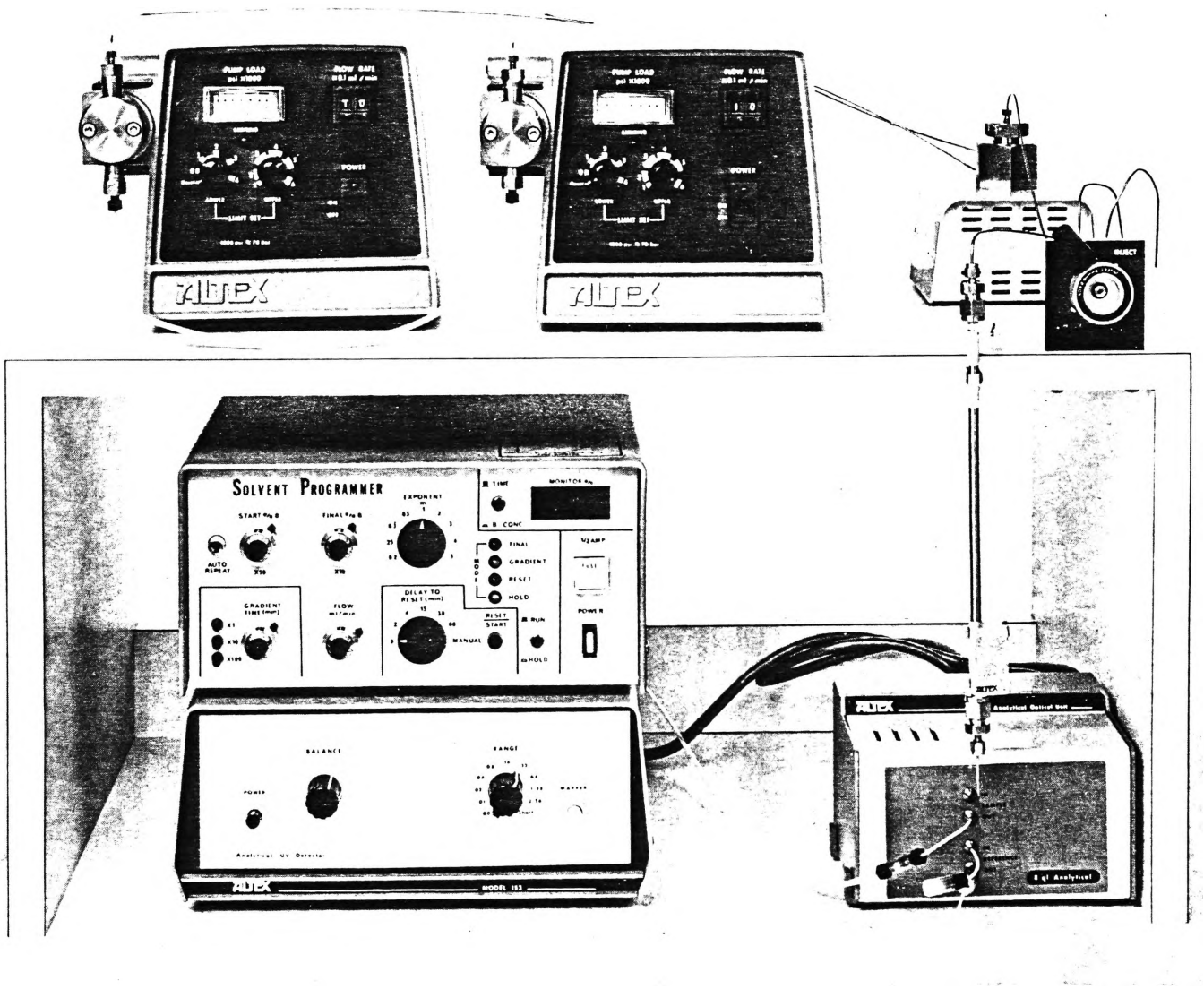
The Model 8500 incorporates a positive displacement syringe pump powered by a stepping motor. It is fitted with a high pressure, stop-flow septumless injector through which samples may be introduced into the mobile phase by means of a syringe.

The Micropak CH10 column (Varian Aerograph) is 25 cm. by 2 mm. i.d. stainless steel packed with 10 micron particles consisting of an octadecyl hydrocarbon bonded to silica. The column's plate number was 1900, measured with benzyl hexadecanoate (palmitate) using a mobile phase of 95% methanol, 5% water.

A low vapour pressure mercury lamp is the source for the UV detector, the 254 nm. line being utilised. The detector is a dual beam, recording differential photometer with a photoconductive detector. All column effluent passes through a 8 ul flow through cell.

FIGURE 3

ALTEX 312 GRADIENT LIQUID CHROMATOGRAPH.



Gradient elutions were performed on an Altex 312 Gradient Chromatograph fitted with a LiChrosorb C18 column and a monochromatic UV detection system. (Figure 3).

The Altex 312 system consists of two reciprocating pumps controlled by a microprocessor. Eight non-linear gradients (4 hyperbolic, 4 parabolic) are available, as well as a linear gradient, ranging over a time span of 1 to 1100 minutes. Hystereses may be incorporated into the gradients. Sample injection is by a 20 ul loop system.

The LiChrosorb C18 column is 25 cm. by 2.1 mm. i.d. stainless steel packed with 5 - 10 micron particles consisting of octadecyl stearate (ODS) bonded to silica. The efficiency of the column was determined using benzyl hexadecanoate (palmitate) using a mobile phase of 95% methanol, 5% water and was found to have 2,400 theoretical plates.

The UV detector incorporated a low vapour pressure mercury lamp (the 254 nm. line being utilised) and an 8 ul flow through cell.

(ii) Reagents.

Fatty Acids: All fatty acids were used as purchased without further purification. The following lists the suppliers.

Acid.	Supplier.
Octanoic (C8:0)	Polyscience Corporation.
Decanoic (C10:0)	Polyscience Corporation.
Undecanoic (C11:0)	Polyscience Corporation.
Dodecanoic (C12:0)	Polyscience Corporation.
Tetradecanoic (C14:0)	Polyscience Corporation.
Hexadecanoic (C16:0)	Polyscience Corporation.
Palmitoleic (C16:1)	Sigma Chemical Co.
Octadecanoic (C18:0)	Polyscience Corporation.
Oleic (C18:1)	Supelco Inc.
Linoleic (C18:2) (cis9,cis12-octadecadienoic acid).	Sigma Chemical Co.
Linolenic (C18:3) (9,12,15-octadecatrienoic acid).	Sigma Chemical Co.
Phytanic	Applied Science Labs. Inc.
n-Eicosanoic (Arachidic) (C20:0)	Sigma Chemical Co.

The other reagents used were purchased from the following suppliers :

Benzyl chloride (Ajax Chemicals) : redistilled (distillation range 177 - 178^{o}C).

n,n-Dimethyl aniline (Hopkin and Williams Ltd.) redistilled (distillation range 192 - 193°C).

Methanol - Spectrosol grade (Ajax Chemicals).

Dry Methanol - prepared by treatment with magnesium activated with iodine.[37]

Diethyl Ether (D.H.A. Pharmaceuticals).

Dry Ether - prepared by removal of peroxides with ferrous ions, and drying with anhydrous $CaCl_2$ then sodium wire.[37]

4-Nitrobenzyl chloride (British Drug Houses Ltd.). Used as purchased.

Heptane - Spectrosol grade (Ajax Chemicals).

Chloroform - Spectrosol grade (Ajax Chemicals).

Dichloromethane - Uvasol grade (Merk).

Extraction Solutions :

Heptane/isopropanol solution[32] for extraction of fatty acids from blood and plasma - 30% heptane mixed with 70% isopropanol.

Methanol/chloroform solution for extraction of fatty acids from dried blood spots - 2:1 (v/v) methanol : chloroform.

(iii) Preparation of Benzyl Dimethyl Anilinium Chloride.[35]

Mix 10.0 gms. of freshly distilled benzyl chloride (distillation range 177 - 178°C) with 9.6 gms. of freshly distilled n,n-dimethyl aniline (distillation range 192 - 193°C). Store in the dark for three days then collect the crystals of benzyl dimethyl anilinium chloride (6.8 gms., m.p. 102 -103°C

Wash the crystals with anhydrous ether.

(iv) Preparation of Benzyl Dimethyl Anilinium Hydroxide Solution.

Mix 1.8 gms. of benzyl dimethyl anilinium chloride with 1.8 gms. of silver oxide, add 15 mls. of spectroscopy grade methanol (Spectrosol grade, Ajax Chemicals) then stir at room temperature for ninety minutes.

Filter off the silver oxide, wash with 10 mls. of methanol, and add the washings to the solution of benzyl dimethyl anilinium hydroxide.

Concentration of solution 0.19 equivalents/litre (titrated against 0.10 N H_2SO_4 to the phenolphthalein end point).

Store in a closed container in a dessicator at less than 10°C. The solution was found to be stable for at least three weeks.

(v) Preparation of 4-Nitrobenzyl Dimethyl Anilinium Hydroxide Solution.

A mixture of 2.8 gms. of n,n-dimethyl aniline and 2.0 gms. of 4-nitrobenzyl chloride in 15 mls. of absolute alcohol was gently warmed to 40°C for two hours, then left to stand at room temperature for several days. The alcohol was removed in vacuo and the crystalline hygroscopic residue of 4-nitrobenzyl dimethyl anilinium chloride[34] was washed with anhydrous ether.

The 4-nitrobenzyl dimethyl anilinium chloride was immediately redissolved in 25 mls. of dry methanol, 2.8 gms. of silver oxide added and the reaction mixture stirred for 90 minutes at room temperature. The slurry was then filtered, the silver oxide washed with 10 mls. of dry methanol which was then added to the clear orange coloured solution of 4-nitrobenzyl dimethyl anilinium hydroxide.

The reagent was found to be 0.14 N on titrating an aliquot of 0.10 N H_2SO_4 (2.0 mls) with the reagent (1.43 mls)

to the phenolphthalein end point.

The reagent was stored in a closed container in a dessicator at less than 10°C and was found to be stable for at least four weeks.

(vi) Preparative Scales of Fatty Acid Derivatisations.

Benzylation.

A typical benzyl-ester preparation is described in detail for palmitic acid.

Palmitic acid (0.195 gms., 0.762 m.moles) was dissolved in methanol and the solution titrated with the benzyl dimethyl anilinium hydroxide reagent (5.25 mls, 1.00 m.moles) to the phenolphthalein end point. All solvent was removed in vacuo, the reaction vessel containing the oily residue sealed and heated for 10 minutes in an oil bath at 110°C. The cooked residue was dissolved in cyclohexane (5 mls) and the solution washed with 1 N HCl (4 lots), 5% Na_2CO_3 solution (2 lots) then water (1 lot).

The solvent was stripped under vacuo and the residue recrystallised from methanol as final purification of the palmitic acid benzyl ester (0.200 gms., 76.1%), m.p. 37°C (Lit. 36°C).[29]

The results of the benzyl ester preparations are summarised in Table 2.

4-Nitrobenzylation.

A typical 4-nitrobenzyl ester preparation is described in detail for palmitic acid.

Palmitic acid (0.232 gms., 0.906 m.moles) was dissolved in methanol and the solution titrated with the 4-nitrobenzyl dimethyl anilinium hydroxide reagent (8.07 mls., 1.13 m.moles) to the bromothymol blue end point. All solvent was removed in vacuo, the reaction vessel containing the red oily residue sealed and heated for 10 minutes in an oil bath at 110°C. The cooked residue was dissolved in a mininum amount of hot methanol, allowed to cool and the resulting solids collected.

Final purification by recrystallising from methanol gave palmitic acid 4-nitrobenzyl ester (0.290 gms., 82%), m.p. 58°C (Lit. 58 - 59°C).

The results of the 4-nitrobenzyl ester preparations are summarised in Table 4.

(vii) Preparation of Solutions of Derivatives Suitable for HPLC.

Benzylation.

The formation of a typical benzyl ester solution prepared for HPLC analysis is described in detail for palmitic acid.

Palmitic acid (0.060 gms., 0.234 m.moles) was dissolved in methanol and the solution titrated with the benzyl dimethyl anilinium hydroxide reagent (1.56 mls, 0.296 m.moles) to the phenolphthalein end point. All solvent was removed in vacuo, the reaction vessel containing the oily residue sealed then heated for 10 minutes in an oil bath at 110°C. The cooked residue was dissolved in cyclohexane (10 mls), the solution washed with 1 N HCl (4 lots), 5% Na_2CO_3 solution (2 lots) and water (1 lot), then dried over anhydrous Na_2SO_4.

This cook was repeated for the acids C8:0, C10:0, C12:0, C14:0, C16:1, C18:0, C18:1, C18:2 and C18:3, portions of the resulting solutions combined and Figure 7 shows the separation of the corresponding benzyl esters by HPLC.

4-Nitrobenzylation.

The formation of a solution of the 4-nitrobenzyl esters of a mixture of saturated fatty acids (Table 6),

suitable for HPLC analysis is described in detail.

An aliquot (1.0 mls, approximately 0.003 milli-equivalents of acid) was titrated with the 4-nitrobenzyl dimethyl anilinium hydroxide reagent to the bromothymol blue end point (0.35 mls, 0.049 milliequivalents). All solvent was removed in vacuo, the reaction vessel containing the oily residue sealed then heated for 10 minutes in an oil bath at 110°C. The cooked residue was dissolved in dichloromethane (2.0 mls), the solution washed with 1 N HCl (4 lots), 5% Na_2CO_3 solution (2 lots) and water (1 lot), then dried over anhydrous Na_2SO_4.

Figure 9 depicts the chromatogram obtained for the products of this procedure.

(viii) Analyses of Physiological Samples.

Derivatisation.

Blood and Plasma Samples. A suitable aliquot of sample (0.50 mls) was diluted with distilled water (1 ml) and acidified with 0.1 N H_2SO_4 (1 ml). An aliquot of an internal standard solution (containing 66.5 ugms. of C20:0) was then added.

The heptane/isopropanol extraction solution (4.0 mls) was used to extract the fatty acids, the upper organic layer collected using a Pasteur pipette. This solution was titrated

with the 4-nitrobenzyl dimethyl anilinium hydroxide reagent (25 drops), to the bromothymol blue end point, in the reaction vessel.

After removal of the solvent in vacuo, the reaction vessel was sealed and immersed in an oil bath, temperature 110°C, for 10 minutes. The residue that resulted after pyrolysis was taken up in 2.0 mls of dichloromethane. This solution was washed with 1 N HCl (4 times), 5% Na_2CO_3 (2 times) and water (once) then dried over anhydrous Na_2SO_4.

Analysis was then by HPLC.

Blood Spot Samples. The fatty acids, in microsamples (100 ul) of blood that had been applied to filter paper and dried, were extracted using the methanol/chloroform mixture (5 mls) by soaking for an hour. Arachidic acid (C20:0) (10 ug) was added as internal standard.

The spots were removed with tweezers after the soaking period and washed with methanol, the washings added to the extraction solution. The composite solution was then titrated, to the bromothymol blue end point, with the 4-nitrobenzyl dimethyl anilinium hydroxide solution (10 drops from a Pasteur pipette) in the reaction vessel.

After removal of solvent in vacuo, the reaction vessel was sealed and immersed in a hot oil bath at 110°C for 10 minutes. The residue was dissolved in dichloromethane (2.0 mls) and the solution washed with 1 N HCl (4 lots), 5%

Na_2CO_3 solution (2 lots) and water (1 lot), then dried over anhydrous Na_2SO_4.

Analysis was performed by HPLC.

(ix) Chromatography.

The analyses of physiological samples were performed on the Altex 312 gradient system after the fatty acids had been derivatised. A fairly complicated gradient system was necessary in order to obtain reasonable separation of the various fatty acid esters. The conditions were :

Instrument : Altex 312 Gradient.
Column : LiChrosorb C18 (25 cm).
Eluent : Methanol/water gradient.
Gradient : Initial - 80% methanol.
Final - 100% methanol.
Exponent - 0.3 (hyperbolic).
Time - 30 minutes.
Instructions - hold at 80% methanol for 7 minutes, then run on the gradient program to 87%. Hold at 87% methanol for 5 minutes, then allow to run the rest of the programme.

Detector Sensitivity : Typically 0.04 aufs.
Chart Speed : 0.5 cms/min.
Injection Size : 20 ul.
Temperature : Room temperature in an air conditioned room (typically 25°C).

DISCUSSION AND RESULTS

A. BENZYL DERIVATIVES OF FATTY ACIDS.

Interest has been shown in the benzyl esters of short chain fatty acids for GLC analysis because they are less volatile than their corresponding methyl esters.[26,39] As well, Politzer et al[33] recognised the increase in sensitivity in liquid chromatographic separations, using ultraviolet absorption detection, of benzyl derivatives of fatty acids.

Preparations by Fischer esterification of carboxylic acids in benzyl alcohol proceed with some difficulty. The steric hinderence imposed by the bulky benzyl group, the effect on the reaction equilibrium of inadequate removal of water from the reaction mixture and the difficulty in removing the high boiling point excess benzyl alcohol without decomposing the esters are thought to be contributing factors.

Politzer et al[33] used a procedure described by White et al[40] which involved condensation of the fatty acid with 1-benzyl-3-p-tolyltriazene followed by a mild thermal decomposition for three hours. Reagent availability and the time factor were the limitations associated with this technique. Some other methods that yield carboxylic acid benzyl esters are available but have restrictions. (The silver salt method is time consuming, and benzylation with phenyldiazomethane[27]

is difficult to implement on a preparative scale because of the explosive nature of the reagent.)

A mild procedure based on the thermal decomposition of benzyl dimethyl anilinium salts of carboxylic acids by refluxing in toluene for an hour was reported by Williams and Halpern[28] to yield excellent results. (Equation 1).

$$R\text{-}COO\overset{\overset{(CH_3)_2}{|}}{\underset{\underset{Ph}{|}}{N}}Bz \xrightarrow{\Delta} RCOOBz + (CH_3)_2NPh \qquad (1)$$

This was the basis of the approach in this study.

The benzyl dimethyl anilinium salts of the carboxylic acids were formed by titrating solutions containing the acids with methanolic benzyl dimethyl anilinium hydroxide to the phenolphthalein end point.

The hydroxide was readily formed by condensing dimethylaniline and benzyl chloride, followed by treatment of a methanolic solution of the resulting benzyl dimethyl anilinium chloride with silver oxide. (Equations 2 and 3).

$$PhN(CH_3)_2 + BzCl \longrightarrow PhN(CH_3)_2Bz^+Cl^- \qquad (2)$$

$$PhN(CH_3)_2Bz^+Cl^- \xrightarrow[CH_3OH]{Ag_2O} PhN(CH_3)_2Bz^+OH^- \qquad (3)$$

The reagent was stored in a sealed container in a desiccator at less than 10^oC and was found to be stable for at least three weeks.

Methods other than refluxing in toluene as the pyrolysis step were sought for several reasons :

· Quantitative analyses of the esters were to be performed by High Performance Liquid Chromatography using a 254 nm. ultraviolet detector. Toluene would absorb this radiation (λ_{max}= 261 nm., ε = 238 $l.mole^{-1}cm.^{-1}$) equally as strongly as the benzyl esters of saturated fatty acids, thus its removal was necessary so as not to interfere with the detection of the separated esters. Removal of the toluene represented another step in the synthesis. No alternative to toluene (B.P. 110^{o}C and inert to this reaction) was readily available.

· Refluxing for one hour was by far the longest step in the synthesis. Reduction in the pyrolysis time to produce a significantly faster analysis was pursued.

Solvent-free pyrolyses of the benzyl dimethyl anilinium salts of carboxylic acids were considered.

A(i) The Reaction.

Preparative scales of formations of benzyl esters of individual fatty acids were conducted in order to :

· Verify the identity of the product.

· Calibrate the HPLC using standard solutions of the benzyl esters.

· Facilitate peak identification in complex chromatograms by spiking methods.

TABLE 2

PREPARATIVE FORMATION OF BENZYL ESTERS

Acid Derivatised	Melting Point of Product (^{o}C)	Melting Point (Lit)[29].	Melting Point of Benzyl Ester.[(a)]	% Yield
Tetradecanoic (C14 : 0) (Myristic)	21	20.5	21	69.4
Hexadecanoic (C16 : 0) (Palmitic)	37	36	37	76.1
Octadecanoic (C18 : 0) (Stearic)	46	45 - 46	46	73.8

(a) Prepared by the method of Williams and Halpern.

Approximately 200 mgm. of acid was dissolved in methanol, titrated with the benzyl dimethyl anilinium hydroxide reagent to the phenolphthalein end point, then all solvent removed in vacuo. The oily residue was heated for 10 minutes at 110°C then dissolved in cyclohexane. The organic solution was washed with acid, Na_2CO_3 solution then water to remove dimethylaniline, the cyclohexane removed in vacuo and the residue recrystallised from methanol.

Identification of the products as benzyl esters was by melting point and comparison of infrared spectra (obtained on a Jasco IRA2 instrument with the sample presentation being in the form of KBr discs) with the corresponding benzyl ester prepared by the method of Williams and Halpern.

Table 2 summarises the results obtained.

A(ii) Temperature of Pyrolysis.

The effect of temperature on the extent of conversion of the benzyl dimethyl anilinium salts of carboxylic acids to benzyl esters by "dry" pyrolysis was investigated.

A standard solution of the salt of hexadecanoic (palmitic) acid was prepared (by dissolving 210 mgms. of the acid in dry methanol, titrating with the benzyl dimethyl anilinium hydroxide reagent to the phenolphthalein end point,

FIGURE 4.

CALIBRATION OF THE VARIAN 8500 ISOCRATIC HPLC.

(BENZYL PALMITATE STANDARDS)

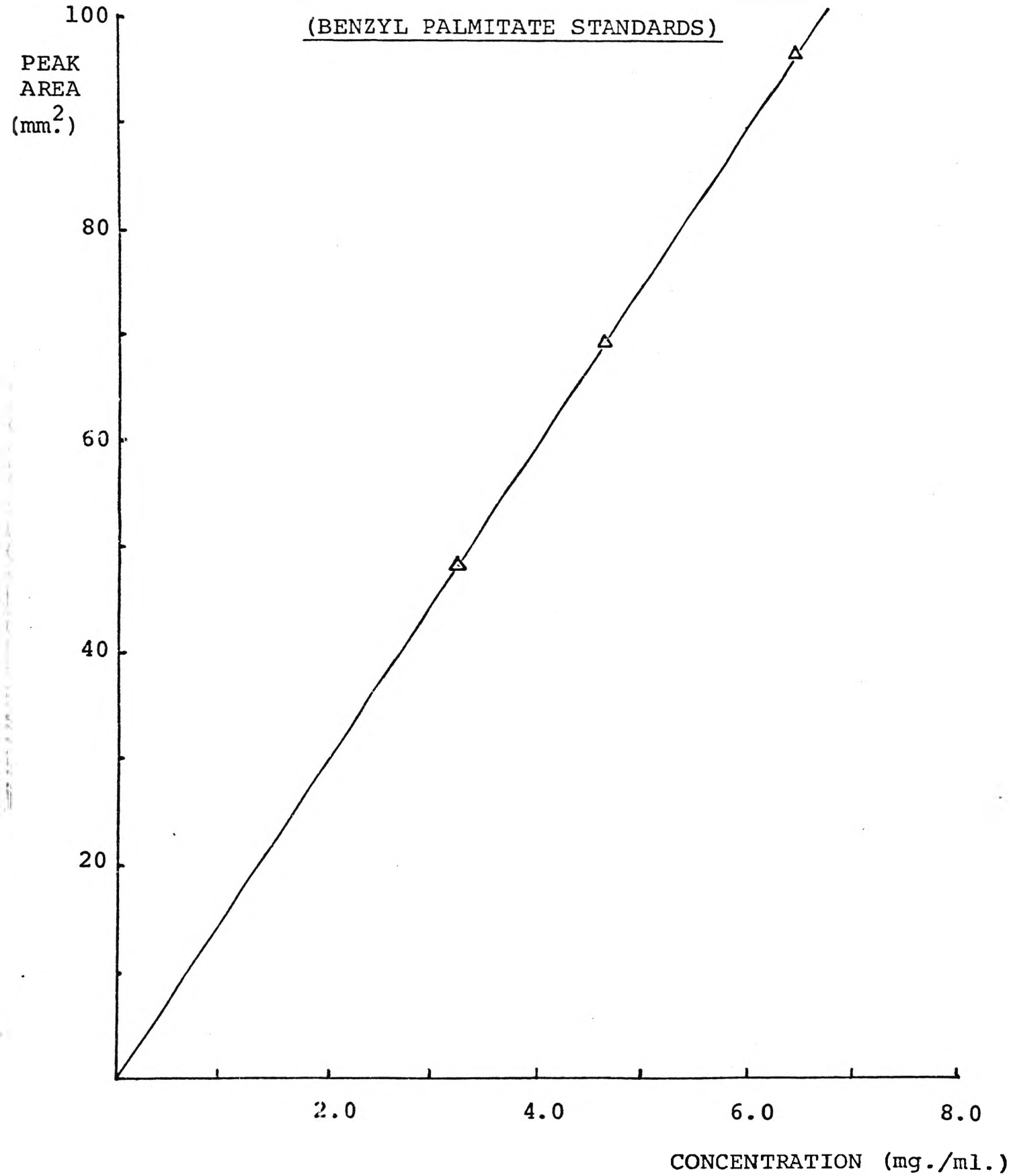


TABLE 3

CALIBRATION OF THE VARIAN 8500 HPLC WITH BENZYL PALMITATE AND EFFECT OF PYROLYSIS TEMPERATURE ON BENZYL ESTER FORMATION.

SOLUTION	PEAK AREA ($mm.^2$) [a]	CONCENTRATION (mg./ml.) [b]	% CONVERSION (average)
Standard (6.5mg./ml.)	96	6.5	
Standard (4.9mg./ml.)	69	4.9	
Standard (3.3mg./ml.)	48	3.3	
110°C. Pyrolysis [c]	83,84	5.6,5.7	98%
120°C. Pyrolysis [c]	84,85	5.7,5.7	100%
135°C. Pyrolysis [c]	77,74	5.2,5.1	91%
150°C. Pyrolysis [c]	70,72	4.7,4.8	84%

[a] Peak Areas reported are the average of three injections (3 ul.) of each solution. Deviations from the average area produced for the injections of each solution were less than 5%.

[b] Concentrations of the sample solutions were determined from the calibration graph (Figure 4).

[c] Duplicate preparations at each pyrolysis temperature were performed and the analytical data reported.

then making the solution up to 100.0 mls. with dry methanol) and aliquots (10.0 mls.) taken and pyrolysed at a particular temperature by the "dry" method. The residues were dissolved in cyclohexane (5.0 mls.), washed with 1 N HCl, 5% Na_2CO_3 solution and water before analysing for the benzyl palmitate concentration by HPLC (theoretical concentration of the cyclohexane solution was 5.68 mg./ml. as benzyl palmitate).

The analysis was conducted using the Varian 8500 Isocratic system fitted with the Micropak CH10 column. (Eluent 95% methanol / 5% water ; flow rate 40 mls./hour ; injection size 3 ul.)

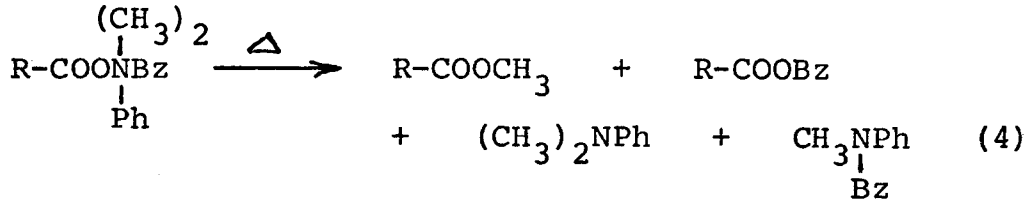
Calibration of the instrument (Figure 4) was conducted using prepared standard solutions of benzyl palmitate.

Table 3 presents the effects of temperature on the degree of conversion to benzyl esters during "dry" pyrolyses, along with the data for calibrations.

All subsequent "dry" pyrolyses of the salts to form benzyl esters were for 10 minutes at 110°C.

A(iii) Methyl Ester Formation.

It was expected that methyl esters of the carboxylic acids would also be formed under the conditions of pyrolysis - the methyl groups of the benzyl dimethyl anilinium ion could compete with the benzyl group. (Equation 4.)



Detection of methyl esters using HPLC fitted with a 254 nm. UV detector would be of little use as the ester absorptivities at these wavelengths are very small (molar extinction coefficients < 100).

Gas/liquid chromatography was used for the detection, and quantitation, of the methyl ester present in the final unwashed cyclohexane solution obtained after pyrolysis at 110°C of the benzyl dimethyl anilinium salt of palmitic acid in an aliquot (10.0 mls.) of the solution previously described.

Gas Chromatography Conditions :

Instrument : Varian Series 200.

Column : OV1 (10%) on Chromsorb W, 2130 mm. X 2.5 mm. (7 ft. X 0.1 inch).

Carrier Gas : Nitrogen, 30 mls./min.

Detector : FID, temperature 240°C, Attenuation Setting 8 X 10^{-9} mA./mV.

Injection Port Temperature : 240°C.

Column Temperature Programme : Initial 140°C, Final 210°C, Rate 6°C/min.

Recorder Chart Speed : 17 mm./min. (40 inches/hour).

One ul. injections of the sample were compared with one ul. injections of a standard methyl palmitate (20% w/v) solution. Simple peak height ratio was used to quantify. (Peak identification was by peak enhancement or "spiking").

	Standard.	Sample.
C16:0 Methyl Ester Retention Time	8.6 mins.	8.6 mins.
Peak Height (1 ul. injection)	63 mms.	15 mms.
Quantity of ester on column.	0.20 ugms.	0.048 ugms. (Calculated).

Thus sample on column = 0.048 ugms. as ester
= 0.046 ugms. as acid.

Concentration of acid converted to methyl ester = 0.046 ugms./ul.
= 0.046 mgms./ml.

Theoretical concentration as acid in the cyclohexane solution
= 4.2 mgs./ml.

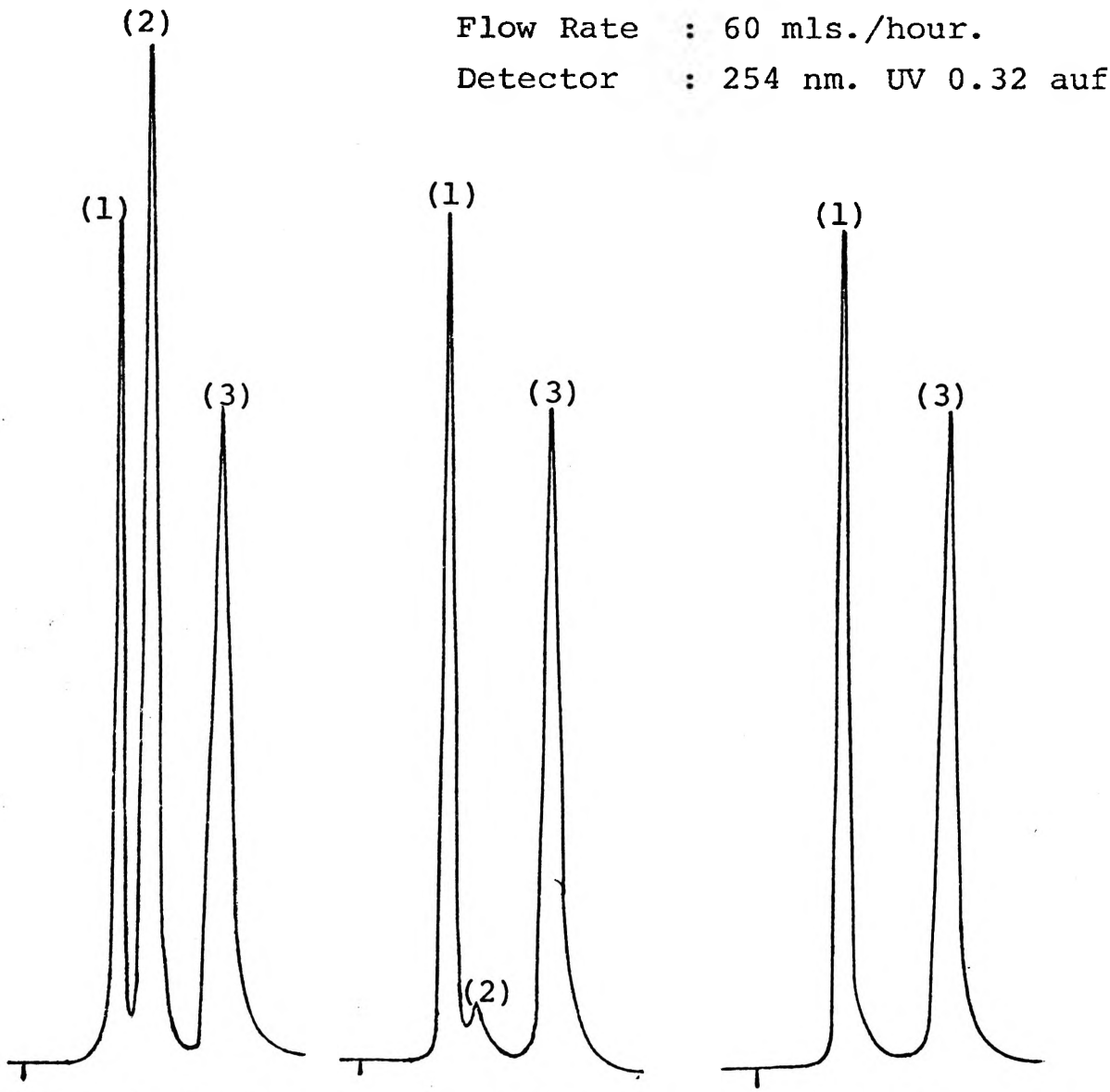
Thus percentage acid converted to the methyl ester = 1.1%

Consequently, some methyl ester formation did occur, under the reaction conditions, but was insignificant.

FIGURE 5.

ACID EXTRACTION (0.1 N HCl) OF DIMETHYLANILINE AFTER PYROLYSIS.

Instrument : Varian 8500.
Column : Micropak CH10 (25 cm.).
Eluent : 85% Methanol
15% Water
Flow Rate : 60 mls./hour.
Detector : 254 nm. UV 0.32 aufs.



Unwashed solution. Solution washed once. Solution washed twice.

(1) Solvent peak.

(2) Dimethylaniline.

(3) Benzyl ester of heptanoic acid.

A(iv) The Washing Process.

The solutions of benzyl esters in cyclohexane were washed with portions of 1 N HCl, 5% Na_2CO_3 solution and distilled water. This was found to be necessary in order to remove the liberated dimethylaniline (Equation 1).

When not extracted, the dimethylaniline tended to overload the analytical column on the HPLC, causing problems with detection of peaks.

Removal of the dimethylaniline was monitored by benzylating heptanoic acid by the "dry" thermal decomposition method, then washing the cyclohexane solution with 0.1 N HCl. Figure 5 shows the HPLC profiles of the cyclohexane solutions produced during a sequence of washings.

The washing process was then incorporated in all subsequent benzylations.

A(v) Chromatography of the Benzyl Esters.

Sensitivity of Analyses by HPLC - 254 nm. UV Detector.

The mininum detectable quantity of benzyl esters on the HPLC using the 254 nm. UV flow through cell detector was determined by making serial dilutions of the C16:0 benzyl ester standard solution. Table 4 summarises the average results obtained for isocratic elutions of duplicate 3 ul.

TABLE 4

DETERMINATION OF THE MININUM DETECTABLE QUANTITY OF PALMITIC ACID BENZYL ESTER BY HPLC

Solution Concentration (mg./ml.)	Ester Quantity Injected (ugms.)	Peak Height (mm.)
3.3	9.9	96
1.7	5.1	46
0.9	2.7	22
0.5	1.5	10
0.3	0.9	4
0.0 (noise)	0.0	2[#]

[#](Measured as peak to trough height).

injections of each of the solutions on the Varian 8500 instrument fitted with the Micropak CH10 column.

Eluent : 95% methanol, 5% water.

Flow : 40 mls./hour.

Detector Sensitivity : 0.02 aufs. (Greater sensitivies could not be utilised due to the resultant noise.)

The mininum detectable quantity, taken as that which produced a peak with height 5 times the noise, was 1.5 ugms. of saturated carboxylic acid benzyl ester.

This is in excellent agreement with the lowest detectable limit for benzyl stearate obtained by Politzer et al in their work[33] on benzyl esters (stated as 1 - 2 ugm.).

Separations.

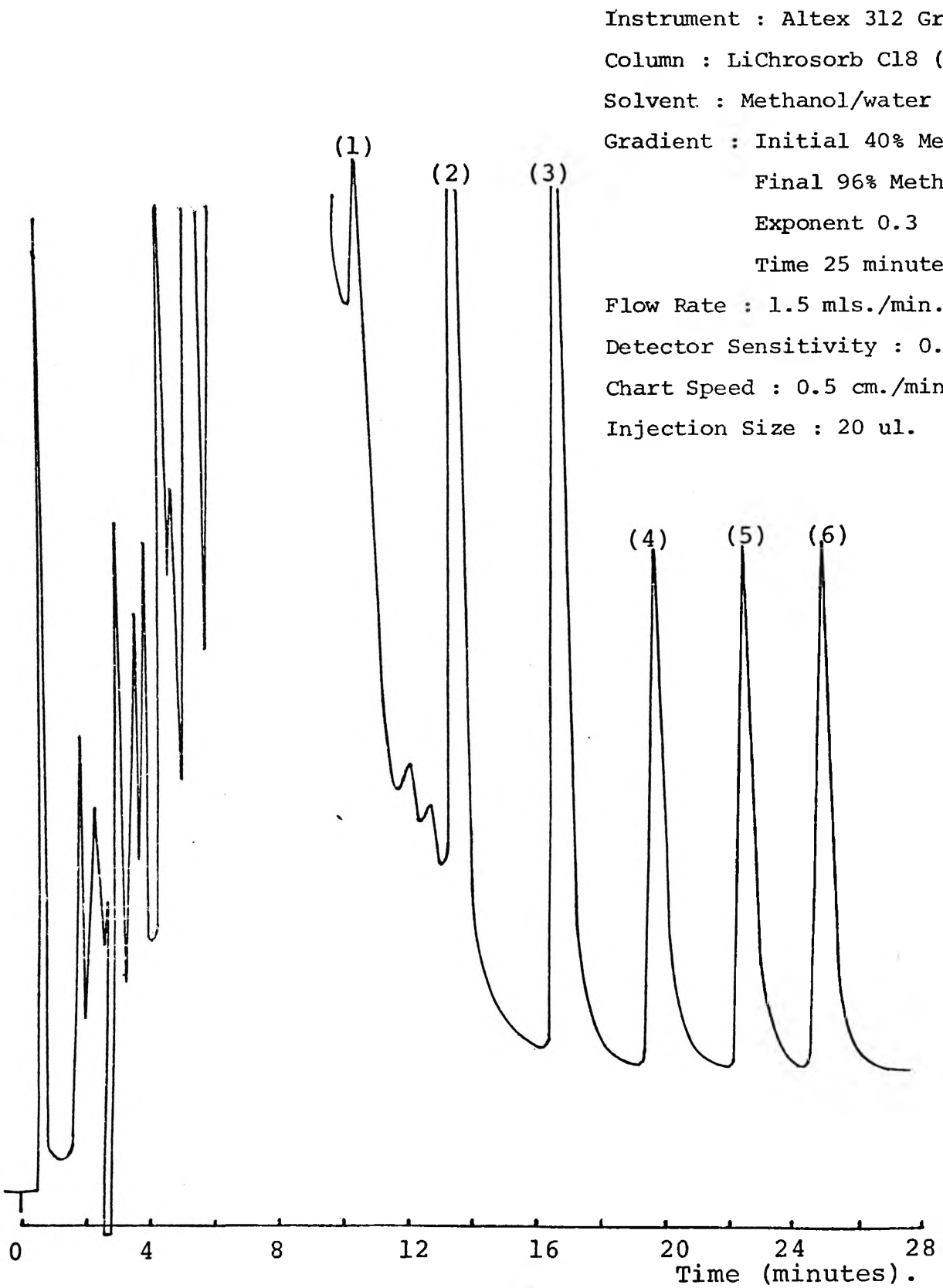
A mixture of benzyl esters of the even carbon numbered saturated carboxylic acids (C8:0 to C18:0) was prepared by combining solutions of the benzyl derivatives formed by treatment of the individual fatty acids. Instrument conditions were determined to give adequate separations.

Isocratic elution from a reverse phase column was unsuccessful so a gradient elution system was developed on the Altex 312 HPLC, fitted with the LiChrosorb C18 reverse phase column, that gave baseline separation.

Figure 6 indicates the separation obtained. Peak

FIGURE 6.

SEPARATION OF BENZYL ESTERS OF SATURATED CARBOXYLIC ACIDS.



(1) Benzyl octanoate (C8:0)

(2) Benzyl decanoate (C10:0)

(3) Benzyl dodecanoate (C12:0)

(4) Benzyl tetradecanoate (C14:0)

(5) Benzyl hexadecanoate (C16:0)

(6) Benzyl octadecanoate (C18:0)

F I G U R E 9

SEPARATION OF 4-NITROBENZYL ESTERS OF SATURATED CARBOXYLIC ACIDS.

Instrument : Altex 312 Gradient.

Column : LiChrosorb C18 (25 cm.).

Solvent : Methanol/Water.

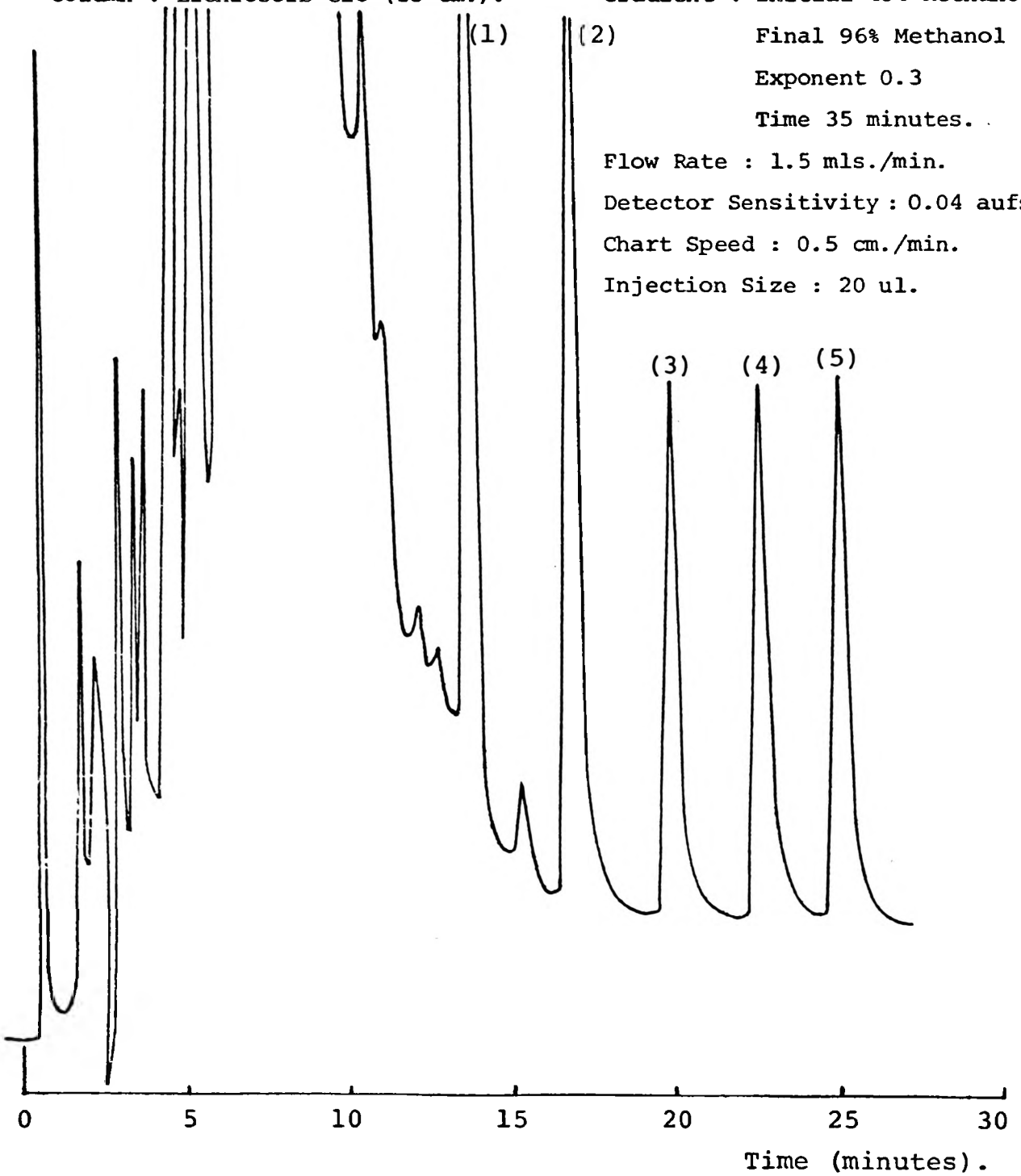
Gradient : Initial 40% Methanol
Final 96% Methanol
Exponent 0.3
Time 35 minutes.

Flow Rate : 1.5 mls./min.

Detector Sensitivity : 0.04 aufs.

Chart Speed : 0.5 cm./min.

Injection Size : 20 ul.



(1) 4-Nitrobenzyl decanoate

(2) 4-Nitrobenzyl dodecanoate

(3) 4-Nitrobenzyl tetradecanoate

(4) 4-Nitrobenzyl hexadecanoate

(5) 4-Nitrobenzyl octadecanoate

identification was performed by spiking.

The C8:0 ester peak was superimposed on the peaks of extraneous compounds produced in the pyrolysis, so that the lowest molecular weight ester able to be quantified was that of C10:0.

However the chromatograms obtained when the benzyl esters of unsaturated fatty acids are added to the benzyl esters of the saturated acids were complicated. Figure 7 indicates the separation of a mixture of the benzyl esters of the carboxylic fatty acids commonly found in physiological fluids. Once again peak identification was performed by spiking.

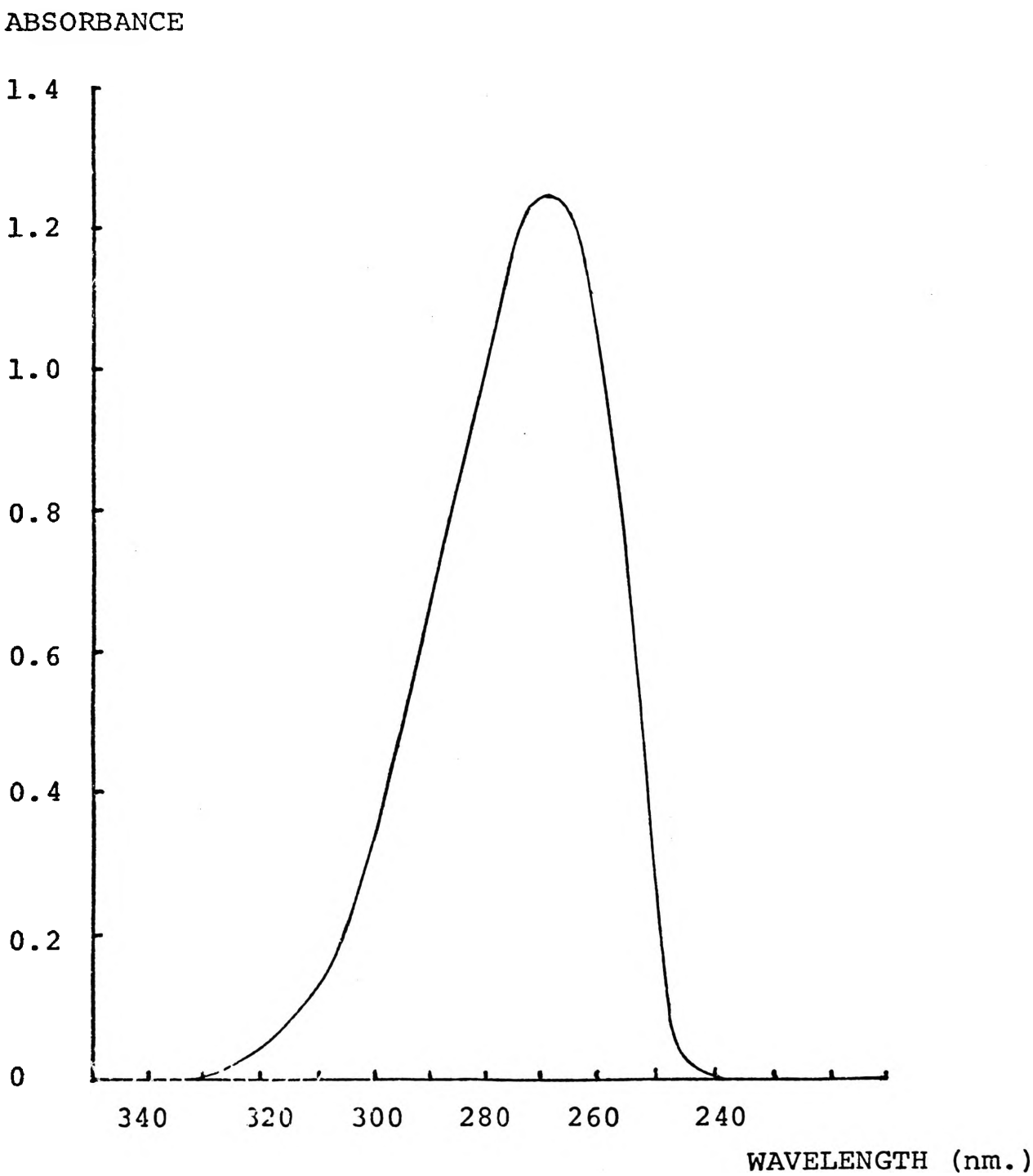
The compound that produced the last peak, purported to belong to the octadecanoic (stearic, C18:0) benzyl ester, was collected and verified as the C18:0 benzyl ester by mass spectrometry. (374, M^+(1%); 283, M^+ - Bz (6%).)

B. 4-NITROBENZYL DERIVATIVES OF CARBOXYLIC ACIDS.

Although it has been shown that conversion of carboxylic acids to their benzyl esters may be performed by thermal decomposition of the benzyl dimethyl anilinium salts of the acids, the resultant analysis by HPLC was not sensitive enough for application to the analysis of free fatty acids in microsamples of physiological fluids.

FIGURE 8.

UV ABSORPTION SPECTRUM OF TETRADECANOIC ACID 4-NITROBENZYL ESTER.



Derivatisation of carboxylic acids to form 4-nitrobenzyl esters was then studied as the auxochromic effect of the nitro group on the benzyl structure greatly increases the absorptivity (or molar extinction coefficient) of UV radiation. e.g.[48]

Benzene	$\mathcal{E}$ = 236	$l.mole^{-1}cm.^{-1}$	λ_{max} = 255 nm.
Toluene	$\mathcal{E}$ = 238	$l.mole^{-1}cm.^{-1}$	λ_{max} = 261 nm.
Nitrobenzene	$\mathcal{E}$ = 7,800	$l.mole^{-1}cm.^{-1}$	λ_{max} = 268 nm.
Nitrotoluene	$\mathcal{E}$ = 11,700	$l.mole^{-1}cm.^{-1}$	λ_{max} = 274 nm.

(Benzene and toluene - only the major peak around 254 nm. for each has been noted.)

UV Absorption of 4-Nitrobenzyl Esters of Carboxylic Acids.

The UV absorption spectrum of a standard solution of the ester of C14:0 (myristic, tetradecanoic acid) (0.032 mg./ml.) in dichloromethane was obtained from a Varian 634 spectrophotometer.

Figure 8 shows the spectrum obtained for the solution in a 1 cm. cell. (Reference : dichloromethane.)

As the same chromophore exists in all the 4-nitrobenzyl derivatives, then for the saturated esters :

λ_{max} = 270 nm.

$\mathcal{E}_{calc.}$ = 14,000

Detection on the HPLC is at 254 nm. From Figure 8 absorbance at 254 nm. is 0.76 . On calculation,

$\mathcal{E}$ = 8600 (λ = 254 nm.)

Initial preparations of the 4-nitrobenzyl esters were conducted using the same conditions established for benzyl ester formations. viz. The 4-nitrobenzyl dimethyl anilinium salt of the carboxylic acid was formed by titrating the solution containing the acid with methanolic 4-nitrobenzyl dimethyl anilinium hydroxide reagent to the phenolphthalein end point.

The reagent was formed by condensing dimethylaniline and 4-nitrobenzyl chloride in ethanol. (Equation 5). The ethanol was stripped under vacuum, the hygroscopic residue of 4-nitrobenzyl dimethyl anilinium chloride[34] washed with anhydrous ether then dissolved in methanol. Hydroxide formation was by treating the methanolic solution with silver oxide. (Equation 6).

$$PhN(CH_3)_2 + NO_2\text{-}BzCl \longrightarrow PhN(CH_3)_2Bz(NO_2)^+Cl^- \quad (5)$$

$$PhN(CH_3)_2Bz(NO_2)^+Cl^- \xrightarrow[CH_3OH]{Ag_2O} AgCl + PhN(CH_3)_2Bz(NO_2)^+OH^- \quad (6)$$

On removal of solids, the hydroxide reagent was found to be 0.14 N on titrating an aliquot of 0.10 N H_2SO_4 (2.0 mls.) with the reagent (0.83 mls.) to the phenolphthalein end point.

The reagent was stored in a sealed container in a dessicator at less than 10°C and was found to be stable for at least four weeks.

TABLE 5

ESTERIFICATION OF CARBOXYLIC ACIDS BY THERMAL DECOMPOSITION OF 4-NITROBENZYL DIMETHYL ANILINIUM SALTS

ESTER OF	MP (^{o}C) (Expt.)	MP (^{o}C) (Lit.[49])	YIELD (%)
C14:0	49	45 - 51.2	74
C16:0	58	58 - 59	82
C18:0	65	64 - 65	77

TABLE 6

STANDARD SATURATED CARBOXYLIC ACID SOLUTION

ACID	QUANTITY WEIGHED (mgms.)	CONCENTRATION (ugms./ml.)
C10:0	8.7	87
C12:0	11.1	111
C14:0	9.8	98
C16:0	9.8	98
C18:0	12.6	126

B(i). The Reaction.

After titration of the carboxylic acid, the solvent was stripped in vacuo, the reaction vessel sealed and the oily residue pyrolysed at 110°C for 10 minutes. Formation of the 4-nitrobenzyl esters by this thermal decomposition method is given by Equation 7.

$$PhN(CH_3)_2Bz(NO_2)^{+-}OOCR \xrightarrow[110^{\circ}C]{\Delta} (NO_2)BzOOCR + PhN(CH_3)_2 \qquad (7)$$

The residue after pyrolysis was dissolved in a mininum amount of hot methanol, allowed to cool overnight and the solids collected and recrystallised from methanol.

Table 5 summarises the results of the 4-nitrobenzyl ester preparations.

The identity of the product formed on derivatisation of palmitic acid was verified as the 4-nitrobenzyl ester by comparison of its infrared spectra (Jasco IRA2 spectrophotometer, KBr disc) with that obtained for 4-nitrobenzyl palmitate formed by condensation of sodium palmitate with 4-nitrobenzyl chloride.[50]

Briefly, palmitic acid (0.25 gms., 1 m.mole) was converted to the sodium salt by neutralisation with alcoholic NaOH (5%). The solution was heated for 4 hours with 4-nitrobenzyl chloride (0.15 gms., 0.9 m.moles) in 5 mls. of alcohol. The ester was collected and recrystallised from methanol

F I G U R E 9

SEPARATION OF 4-NITROBENZYL ESTERS OF SATURATED CARBOXYLIC ACIDS.

Instrument : Altex 312 Gradient.

Column : LiChrosorb C18 (25 cm.).

Solvent : Methanol/Water.

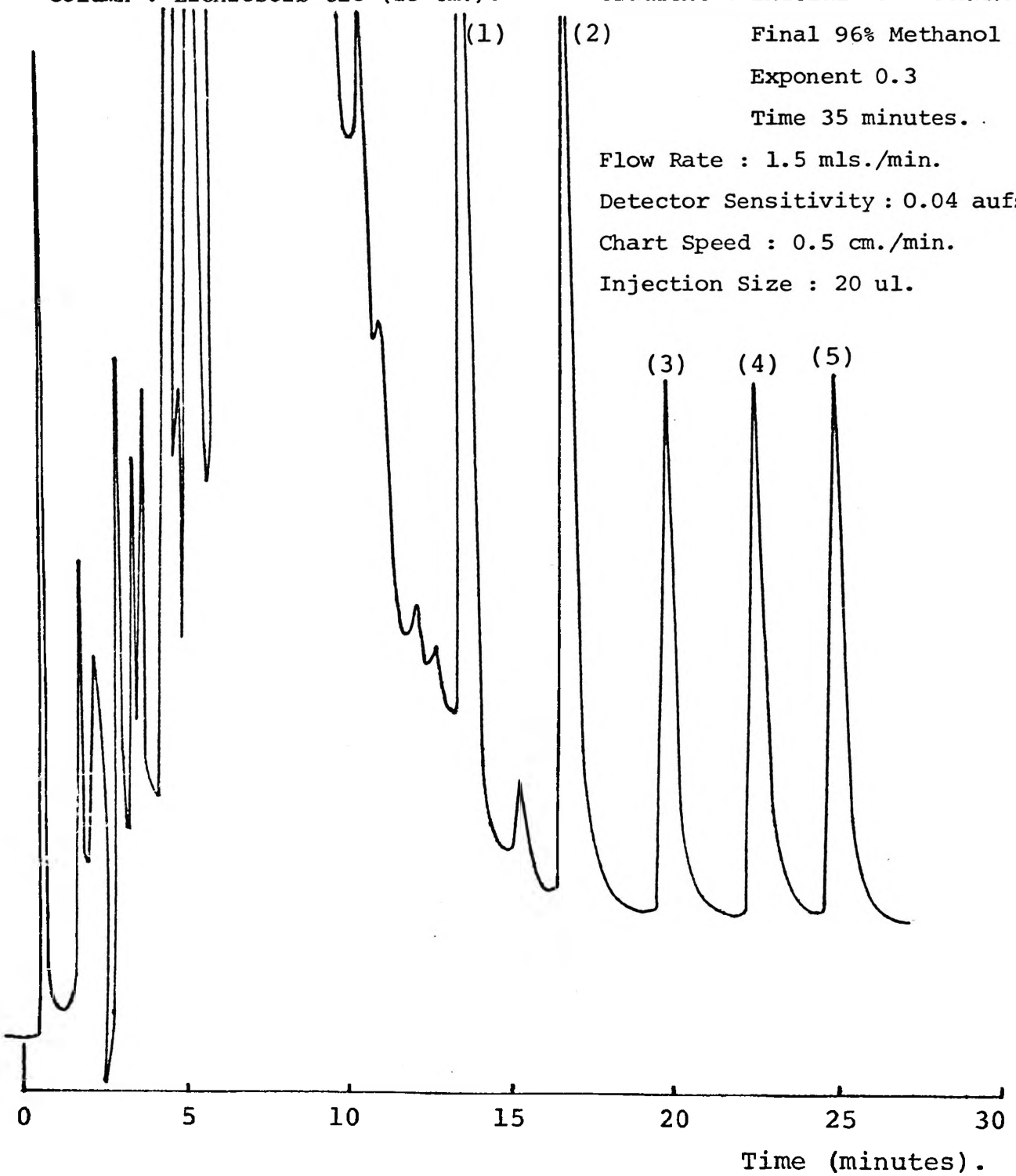
Gradient : Initial 40% Methanol
Final 96% Methanol
Exponent 0.3
Time 35 minutes.

Flow Rate : 1.5 mls./min.

Detector Sensitivity : 0.04 aufs.

Chart Speed : 0.5 cm./min.

Injection Size : 20 ul.



(1) 4-Nitrobenzyl decanoate

(2) 4-Nitrobenzyl dodecanoate

(3) 4-Nitrobenzyl tetradecanoate

(4) 4-Nitrobenzyl hexadecanoate

(5) 4-Nitrobenzyl octadecanoate

(Yield 0.08 gms., 23%; M.P. 58°C).

Optimisation of Reaction Conditions.

A standard solution of a mixture of saturated fatty acids (approximately 10 mgm. of each) was prepared in dichloromethane (100.0 mls.). Aliquots (usually 1.0 ml.) of this solution were then used to quantify the extent of formation of 4-nitrobenzyl esters by thermal decomposition of the acid salts, as well as establishing chromatographic conditions for separation of the resulting esters.

Table 6 lists the quantity of each saturated carboxylic acid in the solution.

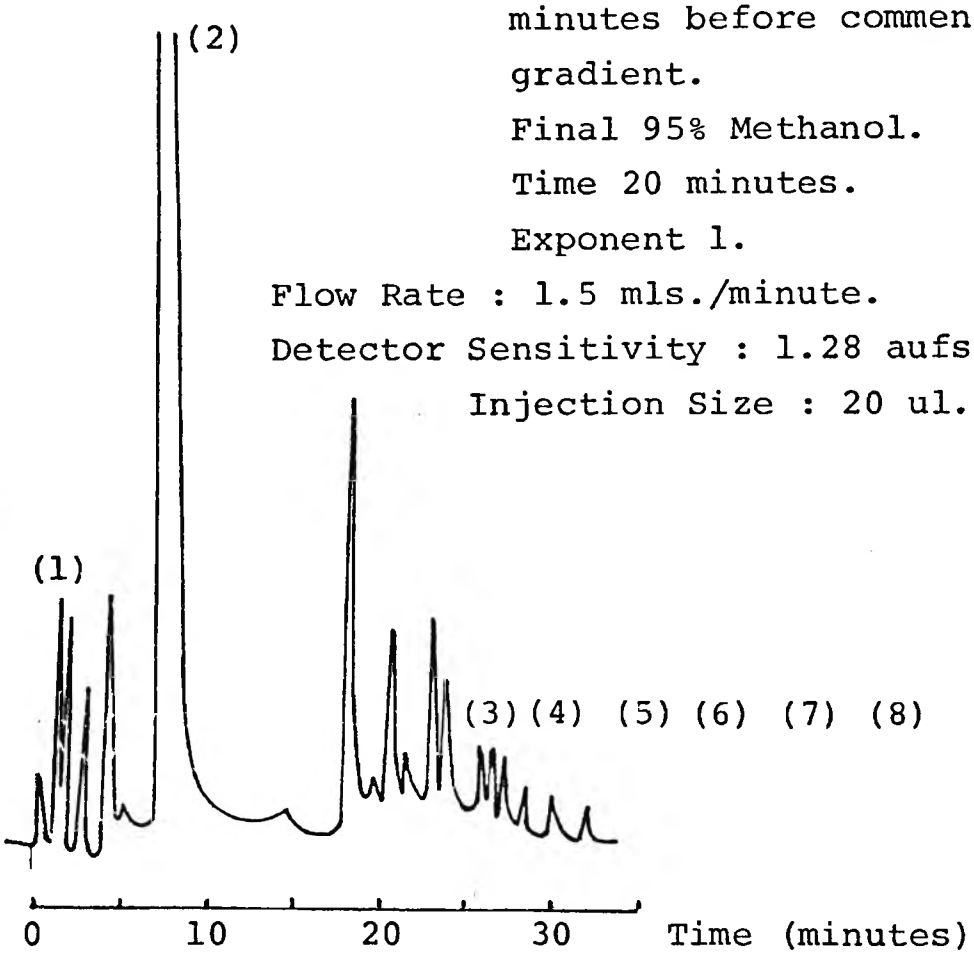
An aliquot of the standard acid solution was titrated with the hydroxide reagent to the phenolphthalein end point and then treated in the usual manner. The residue after pyrolysis was dissolved in dichloromethane (2.0 mls.) and the solution washed with 1 N HCl, 5% Na_2CO_3 solution then water followed by drying over anhydrous Na_2SO_4.

Figure 9 depicts the chromatogram obtained for this solution using the same conditions on the HPLC as for the benzyl esters. Just as occurred in benzylation, components that absorb 254 nm. UV radiation were eluted prior to the esters of interest. The 4-nitrobenzyl ester peaks were identified using spiking techniques.

F I G U R E 1 0

EXTRANEOUS PRODUCTS IN 4-NITROBENZYL DERIVATISATIONS OF CARBOXYLIC ACIDS.

Instrument : Altex 312 Gradient
Column : LiChrosorb C18 (25 cm.)
Eluent : Methanol/Water Gradient
Gradient : Initial 50% Methanol. Hold 12 minutes before commencing gradient.
Final 95% Methanol.
Time 20 minutes.
Exponent 1.
Flow Rate : 1.5 mls./minute.
Detector Sensitivity : 1.28 aufs.
Injection Size : 20 ul.



(1) 4-Nitrophenol
(2) Dimethylaniline
(3) 4-Nitrobenzyl decanoate
(4) 4-Nitrobenzyl undecanoate
(5) 4-Nitrobenzyl dodecanoate
(6) 4-Nitrobenzyl tetradecanoate
(7) 4-Nitrobenzyl hexadecanoate
(8) 4-Nitrobenzyl octadecanoate

Another 4-nitrobenzylation of an aliquot of the standard acid solution, omitting the washing process, gave a chromatogram depicted in Figure 10. Under the conditions indicated it can be seen that there are at least eleven compounds eluted prior to the 4-nitrobenzyl esters. The major peak was due to n;n-dimethylaniline - a byproduct of the reaction. (Equation 7).

(From the titration of the standard acid solution with the hydroxide reagent, the expected dimethylaniline concentration was approximately 3,000 ug./ml. Each ester concentration was approximately 80 ug./ml.)

Another peak was identified, by retention time, as belonging to 4-nitrophenol. This was expected as excess 4-nitrobenzyl dimethyl anilinium hydroxide (0.07 milli-equivalents) was required to titrate the aliquot of acid solution (containing a total of 0.003 milliequivalents of acids) to the phenolphthalein end point. 4-Nitrophenol, then, could just be a product of thermal decomposition of the excess 4-nitrobenzyl dimethyl anilinium hydroxide reagent.

In an attempt to reduce the quantities of the extraneous compounds by reducing the volume of excess titrant, aliquots (1.0 ml.) of the standard acid solution (Table 6) were processed after titrating to alternative indicator end points with the hydroxide reagent.

The effect of the choice of indicator, and thus

TABLE 7

EFFECT OF INDICATOR ON THE PREPARATION OF 4-NITROBENZYL ESTERS OF CARBOXYLIC ACIDS.

Indicator	pH Range	Volume of Titrant to reach End Point.[a]	Relative C16:0 Ester Peak Sizes.[a]
Phenolphthalein	8.2-9.6	0.50 mls.	100
Bromothymol Blue	6.3-7.6	0.35 mls.	100
Methyl Red	4.2-6.2	0.18 mls.	60

[a] Average of duplicates.

TABLE 8

EFFECT OF PYROLYSIS TEMPERATURE ON THE FORMATION OF 4-NITROBENZYL ESTERS OF CARBOXYLIC ACIDS.

Temperature	Relative Peak Area.[a]
90°C	75
100°C	80
110°C	100
120°C	100
140°C	90
150°C	85

[a] Average of duplicates.

the titrant volume, on the extent of 4-nitrobenzylation was determined by comparison of the sizes of the peaks obtained for the palmitic acid ester on HPLC analysis (chromatographic conditions as listed on Figure 9) of the resultant washed dichloromethane solutions.

Results were obtained in duplicate and are summarised in Table 7.

At the normal instrument sensitivities (typically 0.04 aufs), little difference in the sizes of chromatographic peaks due to extraneous compounds in the analysed solutions was observed. However, it was decided to use bromothymol blue as indicator in all subsequent titrations.

B(ii). Effect of Temperature on Thermal Decomposition.

The effect of the pyrolysis temperature on the extent of formation of the 4-nitrobenzyl esters from the corresponding 4-nitrobenzyl dimethyl anilinium salts of the carboxylic acids was determined.

Aliquots (1.0 ml.) of the standard acid solution (Table 6) were titrated to the bromothymol blue end point with the hydroxide reagent, then pyrolysed at particular temperatures, around 110^{o}C, for 10 minutes.

Duplicate preparations were conducted at each

TABLE 9

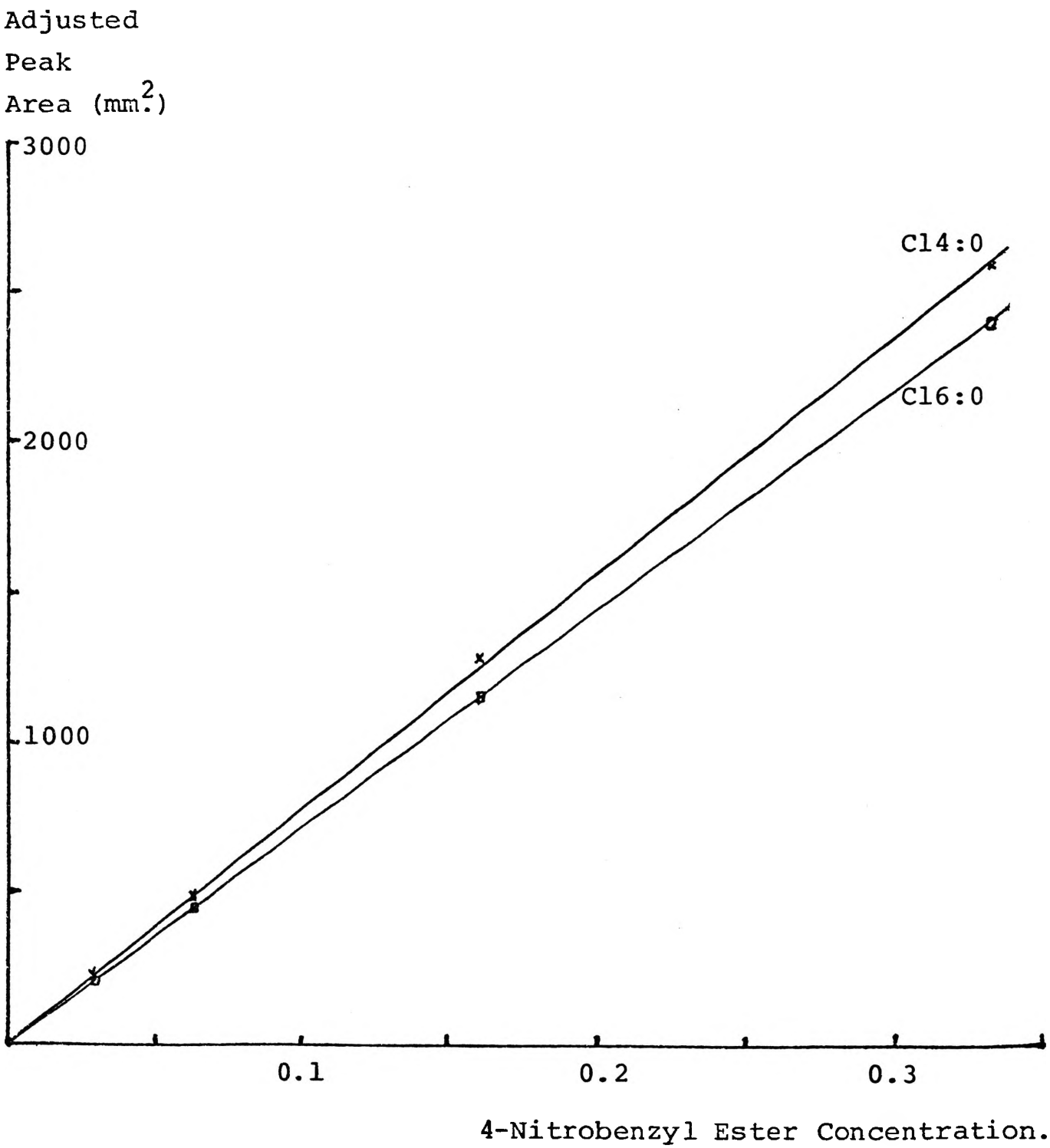
DATA FOR DETERMINING THE EXTENT OF FORMATION OF 4-NITROBENZYL ESTERS.

Acid	Ester Concentration (ug./ml.)	Detector Sensitivity (aufs)	Peak Area ($mm.^2$)	Adj.Peak Area ($mm.^2$) (a)
C14:0	322	0.08	325	2600
	161	0.04	328	1300
	64	0.02	255	510
	32	0.01	260	260
	Sample 1	0.02	300	600
	Sample 2	0.04	297	1188
C16:0	322	0.08	300	2400
	161	0.04	295	1180
	64	0.02	235	470
	32	0.01	240	240
	Sample 1	0.02	270	540
	Sample 2	0.04	263	1052

(a) Adjusted peak areas are relative to a sensitivity of 0.01 aufs.

F I G U R E 1 1

CALIBRATION OF THE HPLC USING 4-NITROBENZYL ESTERS.



temperature, and results obtained from the HPLC chromatograms of the washed dichloromethane solutions (2.0 mls.) of pyrolysis residues. Chromatographic conditions were those previously described. (Figure 9).

Table 8 summarises the results for the palmitic acid peak (peak 4, Figure 9), the relative peak heights being listed. The choice of temperature for pyrolysis was 110°C for all ensuing preparations.

B(iii). Quantitation.

Quantitation of the 4-nitrobenzylation of carboxylic acids by thermal decomposition (10 mins., 110°C) of the 4-nitrobenzyl dimethyl anilinium salts was performed for myristic acid (C14:0) and palmitic acid (C16:0). Aliquots (1.0 ml. and 2.0 mls.) of the standard acid solution (Table 6) were treated and analyses performed on the Altex 312 system fitted with the LiChrosorb C18 column. (Conditions as listed on Figure 9.)

The HPLC was calibrated using standard solutions prepared from recrystallised esters formed by the preparative method. Table 9 lists the chromatographic data from which Figure 11, the calibration graph, was obtained. Sample 1 indicated in Table 9 was a 1.0 ml. aliquot of the standard acid solution, and sample 2 was a 2.0 ml. aliquot. Both were treated and their pyrolysis residues taken up in

T A B L E 1 0

Sample	Acid	Acid mgms.	(a) Quantity m.moles	(a) Theoret.Ester Yield mgms.	(b) Theoretical Conc. mg./ml.	(c) Actual Conc. mg./ml.	Percent Yield
1	C14:0	0.098	0.00043	0.156	0.078	0.075	96.2
1	C16:0	0.098	0.00038	0.150	0.075	0.074	98.7
2	C14:0	0.196	0.00086	0.312	0.156	0.148	95.0
2	C16:0	0.196	0.00076	0.300	0.150	0.144	96.0

Average Yield = 96.5% , Standard Deviation = 1.6

(a) Molecular Weights. C14:0 acid = 228
C14:0 ester = 363
C16:0 acid = 256
C16:0 ester = 391

(b) The final solution containing the esters was made up in 2.0 mls. of dichloromethane.

(c) Actual concentrations were determined from the calibration curves, Figure 11.

dichloromethane (2.0 mls.). HPLC chromatograms were obtained for the washed dichloromethane solutions using the same conditions as for the standards.

Table 10 summarises the results, indicating the suitability of the described experimental procedure as an analytical method.

B(iv). Methyl Ester Formation.

It was expected that methyl esters of the carboxylic acids would also be formed under the conditions of the pyrolysis.

Analysis of the methyl esters using HPLC fitted with a 254 nm. UV detector would be of little use as the methyl ester absorptivities at this wavelength are very small (extinction coefficients < 100).

Gas/liquid chromatography was used for the identification (by spiking) and quantitation of the methyl esters present in the final unwashed solution of the 4-nitrobenzyl esters derived from an aliquot (1.0 ml.) of the standard acid solution (Table 6).

Instrument conditions were :

Instrument : Varian Series 200.

Column : OV1 (10% on Chromsorb W), 2130 mm. X 2.5 mm. i.d. (7 feet X 0.1 inch).

Gas Flow : Nitrogen, 30 mls./minute.

Detector : F.I.D., temperature 240°C.

Injector Temperature : 240°C.

Temperature Programme : Initial 140°C, Final 210°C, Rate 6°C/minute.

Methyl esters were detected in very low concentrations.

Quantitation was done for the C14:0 methyl ester using a simple ratio of peak heights with a standard methyl ester solution.

Sample solution - 2 ul. injection.

C14:0 methyl ester peak height = 2.4 units.

(Detector sensitivity 1 X 10^{-10} mA./mV.)

Standard (20% w./v.) - 1 ul. injection (= 0.2 ug.).

C14:0 methyl ester peak height = 57.0 units.

(Detector sensitivity 8 X 10^{-9} mA./mV.)

From calculation the weight of C14:0 methyl ester in the 2 ul. injection volume was 0.00001 ugms.

Taking into account the C14:0 acid quantity was 100 ugms. in the original 1.0 ml. aliquot of standard acid solution (Table 6), the final solution after pyrolysis was 2.0 mls. in volume and a 2.0 ul. volume of this was injected into the GLC, the computed formation of the methyl ester of C14:0 is 0.1% of the total acid derivatised.

TABLE 11

HPLC. RESPONSE TO

4-NITROBENZYL ESTERS

Concentration of C14:0 Ester. (ug./ml.)	Quantity of Ester on Column. (ug.) (a)	Peak Height ($mm.^2$) (b)
64	1.28	340
32	0.64	173
3.2	0.064	18
1.6	0.032	8
Noise	0.000	3 (c)

(a) The instument was fitted with a 20 ul. loop injection system.

(b) Peak height has been adjusted relative to the detector sensitivity setting of 0.01 aufs.

(c) The noise was measured as trough to peak height.

B(v). Chromatography of the 4-Nitrobenzyl Esters.

Serial dilutions in dichloromethane of the standard solution of the 4-nitrobenzyl esters of myristic and palmitic acids (used for the calibration during quantitation, Table 9) were made in order to determine the sensitivity of detection.

Table 11 summarises the response of the HPLC for the diluted solutions, using the previously listed chromatographic conditions on the Altex 312 system (Figure 9).

The mininum detectable quantity was then calculated to be 0.055 ug. of saturated carboxylic acid 4-nitrobenzyl ester on the column, taken as five times the noise.

Separations and Choice of Internal Standard.

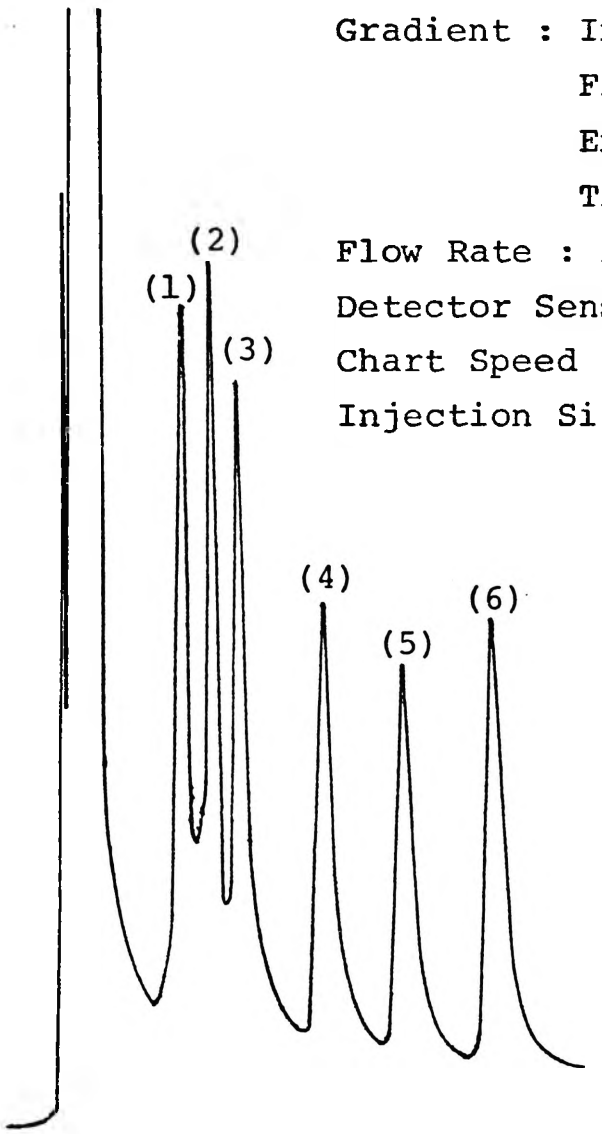
The formation of carboxylic acid 4-nitrobenzyl esters by thermal decomposition of the 4-nitrobenzyl dimethyl anilinium salts was shown to go essentially to completion. However calibration of the HPLC on each analysis was a tedious exercise so an internal standard method of quantitation was established.

It was thought that a carboxylic acid would be ideal as it could be added to the sample prior to derivitisation. In this way errors may be reduced.

F I G U R E 1 2

UNDECANOIC ACID AS INTERNAL STANDARD.

Instrument : Altex 312 Gradient.
Column : LiChrosorb C18 (25 cm.).
Gradient : Initial 40% Methanol
Final 96% Methanol
Exponent 0.3
Time 35 minutes.
Flow Rate : 1.5 mls./minute.
Detector Sensitivity : 0.04 aufs.
Chart Speed : 0.2 cms./minute.
Injection Size : 20 ul.



(1) 4-Nitrobenzyl decanoate

(2) 4-Nitrobenzyl undecanoate

(3) 4-Nitrobenzyl dodecanoate

(4) 4-Nitrobenzyl tetradecanoate

(5) 4-Nitrobenzyl hexadecanoate

(6) 4-Nitrobenzyl octadecanoate

The original choice for an internal standard was undecanoic acid (C11:0) as it was readily available, and does not occur naturally in physiological fluids.[2] Consequently it would not interfere with analyses of the acids in those fluids.

A standard solution of the C11:0 acid was prepared (88 ug./ml.) and an aliquot (1.0 ml.) added to an aliquot (1.0 ml.) of the standard acid mixture solution (Table 6). A solution of the nitrobenzyl esters in dichloromethane was prepared by the method previously established and after the washing process a chromatogram run. Figure 12 depicts the type of chromatogram obtained. The C11:0 ester peak could not be completely resolved, even though good separation was attainable for the derivatives of the even numbered saturated acids.

Consequently the C11:0 acid was rejected as an internal standard. Pentadecanoic acid (C15:0) was not tried as it would present the same problem, particularly in the presence of the unsaturated acid esters.

Although it is a naturally occurring acid, n-eicosanoic acid (arachidic acid - straight chained C20:0 that occurs in peanuts, vegetables and fish oil[38]) was studied as a possible internal standard. References[1] indicate that the level of acids greater than C18 are minimal in humans as free acids, so interferences in analyses of physiological fluids would be unlikely.

TABLE 12

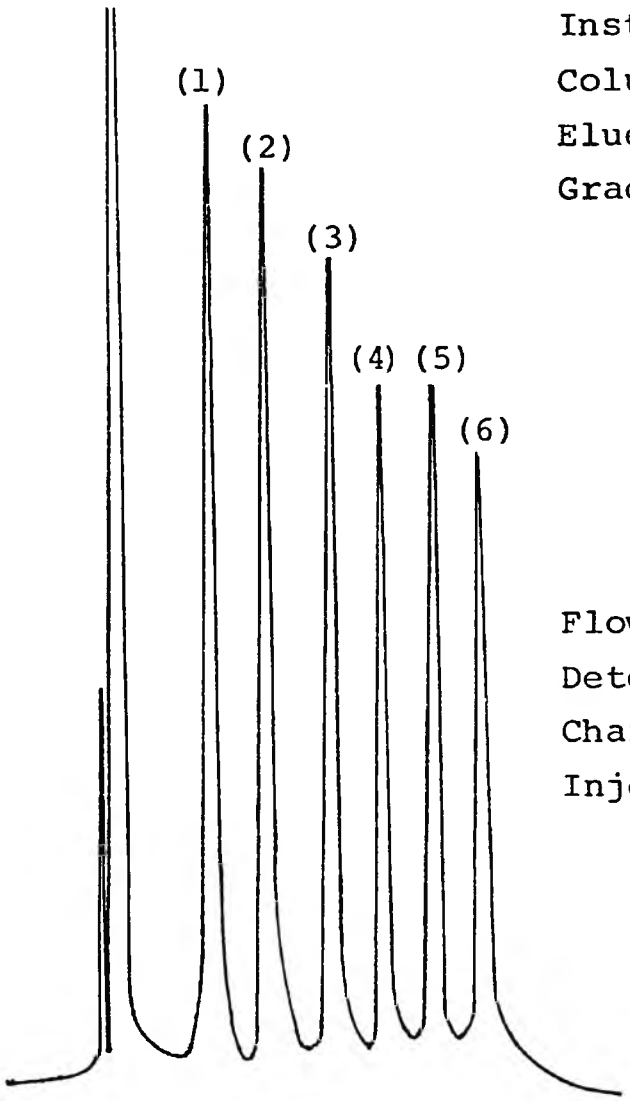
STANDARD UNSATURATED CARBOXYLIC ACID SOLUTION.

Acid	Quantity Weighed (mgms.)	Concentration (ugms./ml.)[a]
C16:1	11.7	117
C18:1	13.6	136
C18:2	5.5	55
C18:3	4.6	46
Phytanic Acid	10.5	105

(a) The acids were dissolved and made up to 100.0 mls. in dichloromethane.

F I G U R E 1 3

n-EICOSANOIC ACID (ARACHIDIC ACID, C20:0) AS INTERNAL STANDARD - SATURATED ACIDS.



Instrument : Altex 312 Gradient.
Column : LiChrosorb C18 (25 cm.).
Eluent : Methanol/Water gradient.
Gradient : Initial 80% Methanol
Final 100% Methanol
Exponent 0.3
Time 30 minutes.
Hold 80% for 7 minutes
run to 87% then hold
5 minutes. Continue
gradient.
Flow Rate : 1.5 mls./minute.
Detector Sensitivity : 0.08 aufs.
Chart Speed : 0.2 cms./minute.
Injection Size : 20 ul.

(1) 4-Nitrobenzyl decanoate (C10:0)

(2) 4-Nitrobenzyl dodecanoate (C12:0)

(3) 4-Nitrobenzyl tetradecanoate (C14:0)

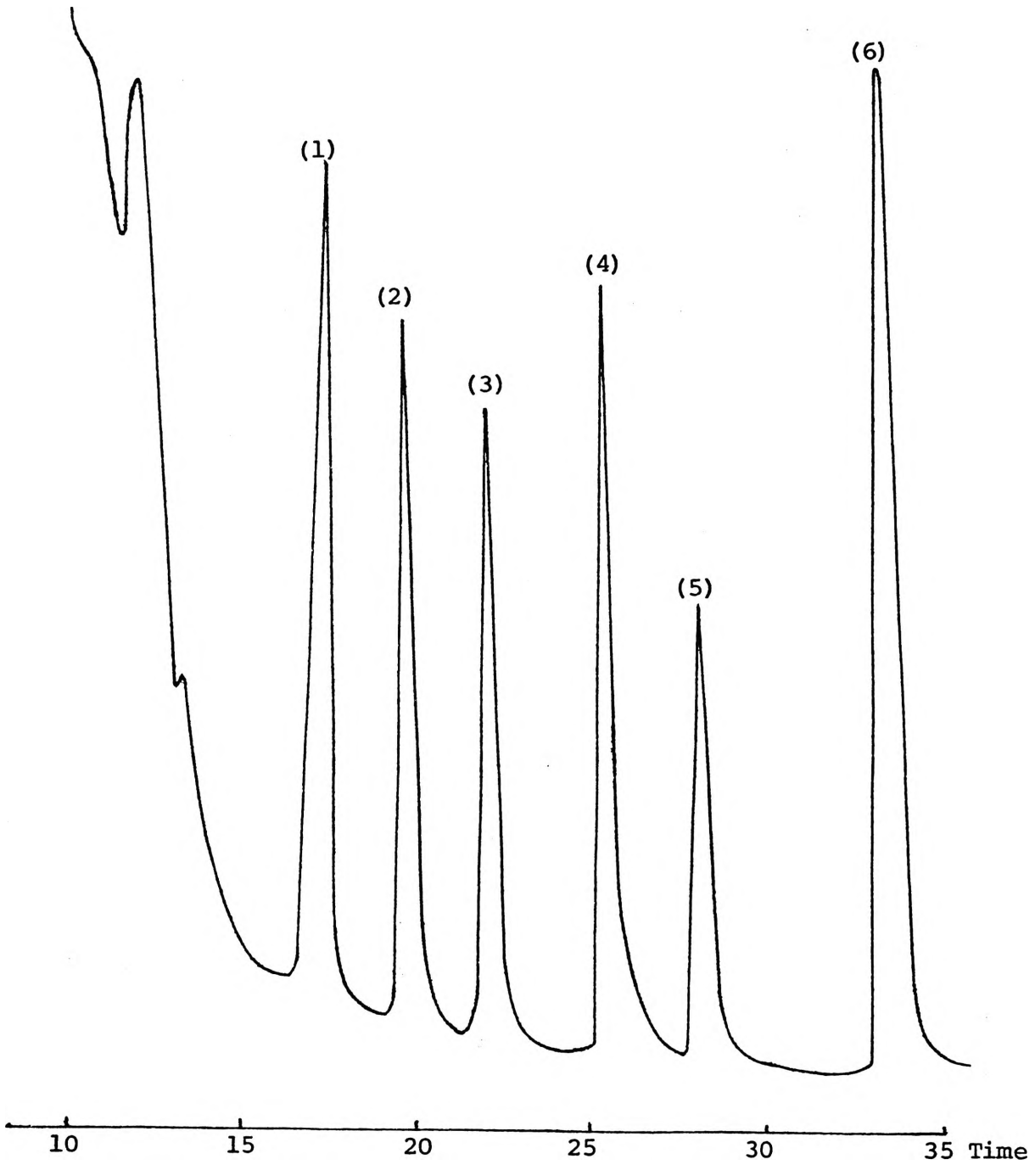
(4) 4-Nitrobenzyl hexadecanoate (C16:0)

(5) 4-Nitrobenzyl octadecanoate (C18:0)

(6) 4-Nitrobenzyl n-eicosanoate (C20:0)

F I G U R E 1 4

n-EICOSANOIC ACID AS INTERNAL STANDARD - UNSATURATED ACIDS + PHYTANIC ACID.



Instrument : Altex 312 Gradient. Column : LiChrosorb C18 (25 cm.).

Eluent : Methanol/Water gradient.

Gradient : Initial 80% Methanol, Final 100% Methanol, Exponent 0.3
Time 30 minutes. Hold 80% for 7 minutes, run to 87% then hold 5 minutes. Continue gradient.

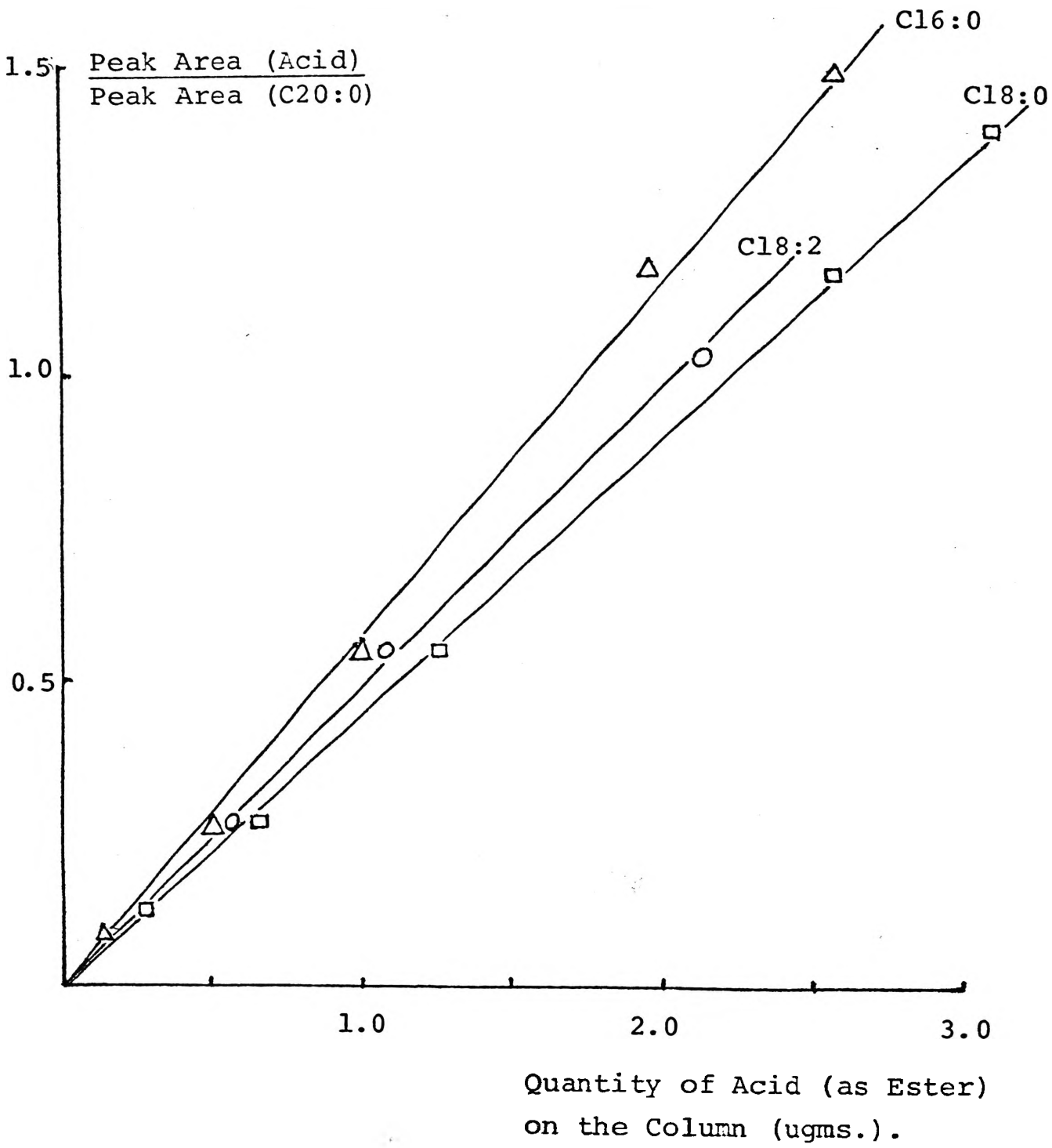
Flow Rate : 1.5 mls./minute. Detector Sensitivity : 0.04 aufs.

Chart Speed : 0.5 cms./minute. Injection Size : 20 ul.

(1) 4-Nitrobenzyl linolenoate (C18:3)
(2) 4-Nitrobenzyl palmitolenoate (C16:1)
(3) 4-Nitrobenzyl linoleate (C18:1)
(4) 4-Nitrobenzyl oleate (C18:1)
(5) 4-Nitrobenzyl phytanate
(6) 4-Nitrobenzyl n-eicosonoate (C20:0)

F I G U R E 1 5

SUITABILITY OF C20:0 AS AN INTERNAL STANDARD.



The same methods as used for C11:0 acid were used. Figure 13 shows the typical chromatogram with the C20:0 acid as an internal standard in the mixture of saturated fatty acids. (Modifications to the chromatographic conditions of Figure 9 were necessary to obtain a reasonable peak shape for the C20:0 ester.)

To ensure that adequate peak separation would result on HPLC analyses of their 4-nitrobenzyl esters, a solution containing known quantities of the naturally occurring unsaturated fatty acids, plus phytanic acid, was prepared. Table 12 shows the composition of the solution.

Aliquots of this solution were used later for quantitation studies using arachidic acid as an internal standard.

Figure 14 depicts the chromatogram obtained after the acids contained in an aliquot of the solution containing mainly unsaturated acids (Table 12) were nitrobenzylated. Arachidic acid was added as an internal standard.

Quantitation.

The suitability of arachidic acid as an internal standard for quantitation of several common fatty acids is demonstrated in Figure 15.

The use of arachidic acid as an internal standard

TABLE 13

ARACHIDIC ACID (C20:0) AS INTERNAL STANDARD - SATURATED ACIDS.

Acid	Quantity Pyrolysed (ug.)	Peak Area# (mm^2)	Area/Unit Mass of Acid (mm^2/ugm.)	Constant
C12:0	28	91	3.25	1.79
	56	185	3.30	1.81
	111	375	3.38	1.84
C14:0	25	72	2.88	1.58
	49	150	3.06	1.68
	98	295	3.01	1.65
C16:0	25	66	2.64	1.45
	49	136	2.78	1.53
	98	274	2.79	1.53
C18:0	32	68	2.13	1.17
	63	136	2.16	1.19
	126	275	2.18	1.20
C20:0	133	248	1.86	
	133	245	1.84	
	133	235	1.77	
			Average = 1.82	

Peak Area taken as Peak Height X Width at Half Height.

for analyses of free fatty acids in physiological fluids necessitated in situ 4-nitrobenzylation of specific quantities of arachidic acid with known quantities of all the normal fatty acids. Correlations of the detector responses for those quantities of normal acids with the responses for arachidic acid quantities were then determined.

For the saturated fatty acids, aliquots (0.25, 0.5 and 1.0 mls.) of the standard acid solution (Table 6) were combined with an aliquot (1.0 ml.) of a prepared arachidic acid solution (133 ugm./ml. in ethanol) and then derivatised by the established method. Data from the chromatograms (instrument conditions as listed in Figure 13) obtained from analysis of the resulting dichloromethane solutions is summarised in Table 13.

The column labelled "Constant" is the ratio of the peak area per mass of acid derivatised to the peak area per mass of arachidic acid added as internal standard. The "Constant" may be used for the quantitation of a particular fatty acid in a sample by :

$$\text{Mass of fatty acid in the sample} = \frac{(\text{Mass of C20:0 added}) \times (\text{Area of fatty acid peak})}{(\text{Area of C20:0 peak}) \times (\text{Constant from the table})}$$

(Appendix 1 shows the derivation of the above equation).

TABLE 14

ARACHIDIC ACID (C20:0) AS INTERNAL STANDARD - UNSATURATED ACIDS PLUS PHYTANIC ACID.

Acid	Quantity Pyrolysed (ug)	Peak Area# ($mm.^2$)	Area/Unit Mass of Acid ($mm.^2/ugm.$)	Constant
C16:1	59	110	1.87	1.00
	117	226	1.93	1.03
	234	452	1.93	1.03
C18:1	68	149	2.19	1.17
	136	290	2.13	1.14
	272	585	2.15	1.15
C18:2	28	69	2.47	1.32
	55	138	2.51	1.34
	109	265	2.43	1.30
C18:3	23	62	2.69	1.44
	46	123	2.67	1.43
	92	243	2.64	1.41
Phyt-	53	85	1.61	0.86
anic	105	163	1.55	0.83
	210	330	1.57	0.84
C20:0	133	250	1.88	
	133	245	1.84	
	133	252	1.89	
			Average = 1.87	

#Peak Area taken as Peak Height X Width at Half Height.

TABLE 15

CONSTANTS WHEN USING ARACHIDIC ACID (C20:0) AS INTERNAL STANDARD.

Fatty Acid	Constant
C12:0	1.81
C14:0	1.65
C16:0	1.53
C16:1	1.03
C18:0	1.19
C18:1	1.15
C18:2	1.32
C18:3	1.43
Phytanic Acid	0.84

This method enables adjustment of the quantity of the internal standard to suit the sample that is to be analysed.

Table 14 summarises the data obtained, using the same procedure, for those fatty acids in the solution of mainly unsaturated acids (Table 12).

Table 15 lists the average of the constants for each of the acids.

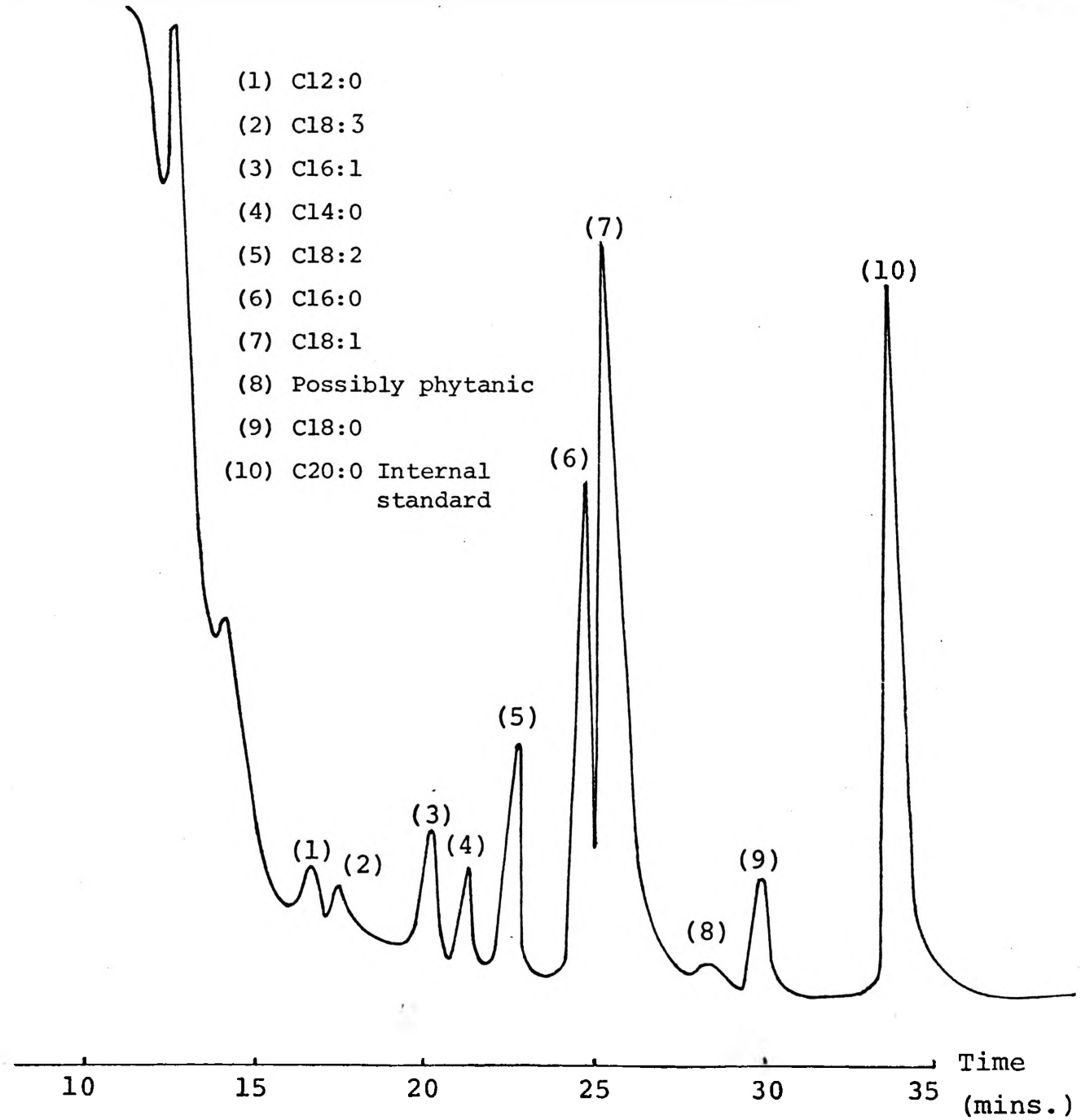
The chromatographic data for the C20:0 was that obtained for six separate cooks of the same quantity and demonstrates the reproducibility of the method. In each case an aliquot (1.0 ml.) of a standard solution (133 ugm./ml.) of the C20:0 was added to the sample solution as the internal standard.

For these six cases, the HPLC area/mass of C20:0 acid had an average of 1.85 with a relative standard deviation of 2.3% .

C. ANALYSIS OF FREE FATTY ACIDS IN BLOOD.

An application of the foregoing derivatisations of carboxylic acids is to determine the total and individual free fatty acid levels in whole blood and sera. As previously indicated the expected total free fatty acid content is

FIGURE 16. CHROMATOGRAPHIC ANALYSIS OF FREE FATTY ACIDS, AS 4-NITROBENZYL ESTERS, EXTRACTED FROM HUMAN BLOOD PLASMA.



Instrument : Altex 312 Gradient. Column : LiChrosorb C18 (25 cm.).

Eluent : Methanol/Water gardient.

Gradient : Initial 80% Methanol, Final 100% Methanol. Exponent 0.3 . Time 30 minutes. Hold 80% for 7 minutes, run to 87%, then hold 5 minutes. Continue Gradient.

Flow Rate : 1.5 mls./minute. Detector Sensitivity : 0.04 aufs.

Chart Speed : 0.5 cms./minute. Injection Size : 20 ul.

TABLE 16

BLOOD PLASMA[a] - WOLLONGONG DISTRICT HOSPITAL.

Acid	Peak Area[b] (mm^2)	Quantity of Acid[c] (ug.)	Percentage
C20:0[d]	416	66.5	
C18:0	37.5	5.0	5.5
C18:1	275	38.2	42.4
C16:0	222	23.2	25.7
C18:2	85	10.3	11.4
C14:0	26	2.5	2.8
C16:1	50	7.8	8.6
C18:3	12.5	1.4	1.6
C12:0	21.0	1.8	2.0
		Total = 90.2 ugms.	

(a) Sample Size = 0.5 mls. Thus total free fatty acid content is 90.2 ugms./0.5 mls. = 18.0 mg.%

(b) Taken as Peak Height X Width at Half Height.

(c) Calculated using the equation derived for the internal standard technique (page 44).

(d) Data listed in reverse order of component elution.

approximately 20 mg.%. The normal blood sample may vary from 20 ul. to 1000 ul.; the total free fatty acid range is, then, 4 ug. to 200 ug.

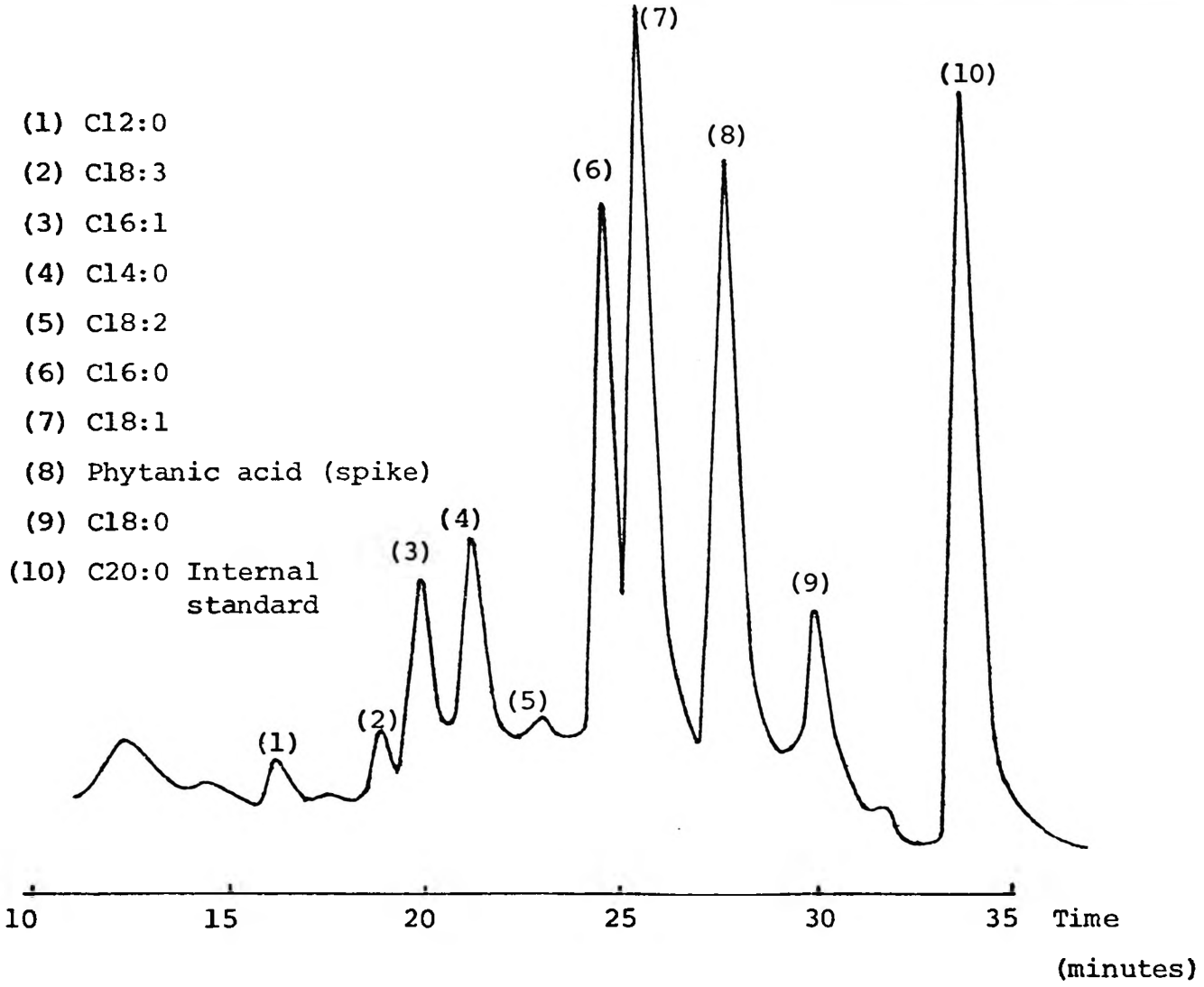
The mininum detectable quantity of the 4-nitrobenzyl esters of saturated fatty acids (0.055 ug. on the column) was much smaller than that for the benzyl esters (1.5 ug. on the column) on analysis by HPLC fitted with a 254 nm. UV detector. Consequently all analyses were done by forming the 4-nitrobenzyl derivatives of the free fatty acids.

C(i). Quantitative Analyses of Free Fatty Acids in Blood and Blood Plasma.

Analysis of the free fatty acid contents of blood plasma (supplied by Wollongong District Hospital) was conducted by extraction of the free fatty acids from an acidified sample (500 ul.) using the heptane/isopropanol extraction solution.[31] An aliquot of the internal standard (arachidic acid, C20:0) solution was added to the sample prior to extraction.

Figure 16 shows the chromatogram obtained for a 20 ul. injection of the resulting solution after derivatisation. Peak identification was by the spiking technique. Table 16 summarises the results obtained from the chromatogram. The total free fatty acids, 18.0 mg.%, is a reasonable match to the expected 20 mg.%.

FIGURE 17. CHROMATOGRAPHIC ANALYSIS OF FREE FATTY ACIDS, AS 4-NITROBENZYL ESTERS, EXTRACTED FROM HUMAN BLOOD SPIKED WITH PHYTANIC ACID.



Instrument : Altex 312 Gradient. Column : LiChrosorb C18 (25 cm.).

Eluent : Methanol/Water.

Gradient : Initial 80% Methanol. Final 100% Methanol.

Exponent 0.3 . Time 30 minutes.

Hold 80% for 7 minutes, run to 87% then hold 5 minutes. Continue gradient.

Flow Rate : 1.5 mls./minute.

Detector Sensitivity : 0.04 aufs.

Chart Speed : 0.5 cms./minute.

Injection Size : 20 ul.

TABLE 17

FRESH BLOOD.

Acid[a]	Peak Area[b] (mm^2)	Quantity of Acid[c] (ug.)	Percentage
C20:0	432	66.5	
C18:0	77	10.2	9.6
Phytanic	292	53.5	
C18:1	368	49.3	46.4
C16:0	196	20.0	18.8
C18:2	6	0.7	0.7
C14:0	78	7.4	7.0
C16:1	100	14.9	14.0
C18:3	20	2.2	2.1
C12:0	18	1.5	1.4

Initial Blood Total = 106.2 ugms.

(a) Sample Size = 0.5 mls. Thus total free fatty acid content is 106.2 ugms./0.5 mls. (excluding phytanic) = 21.2 mg.%

(b) Taken as Peak Height X Width at Half Height.

(c) Calculated using the equation derived for the internal standard technique (page).

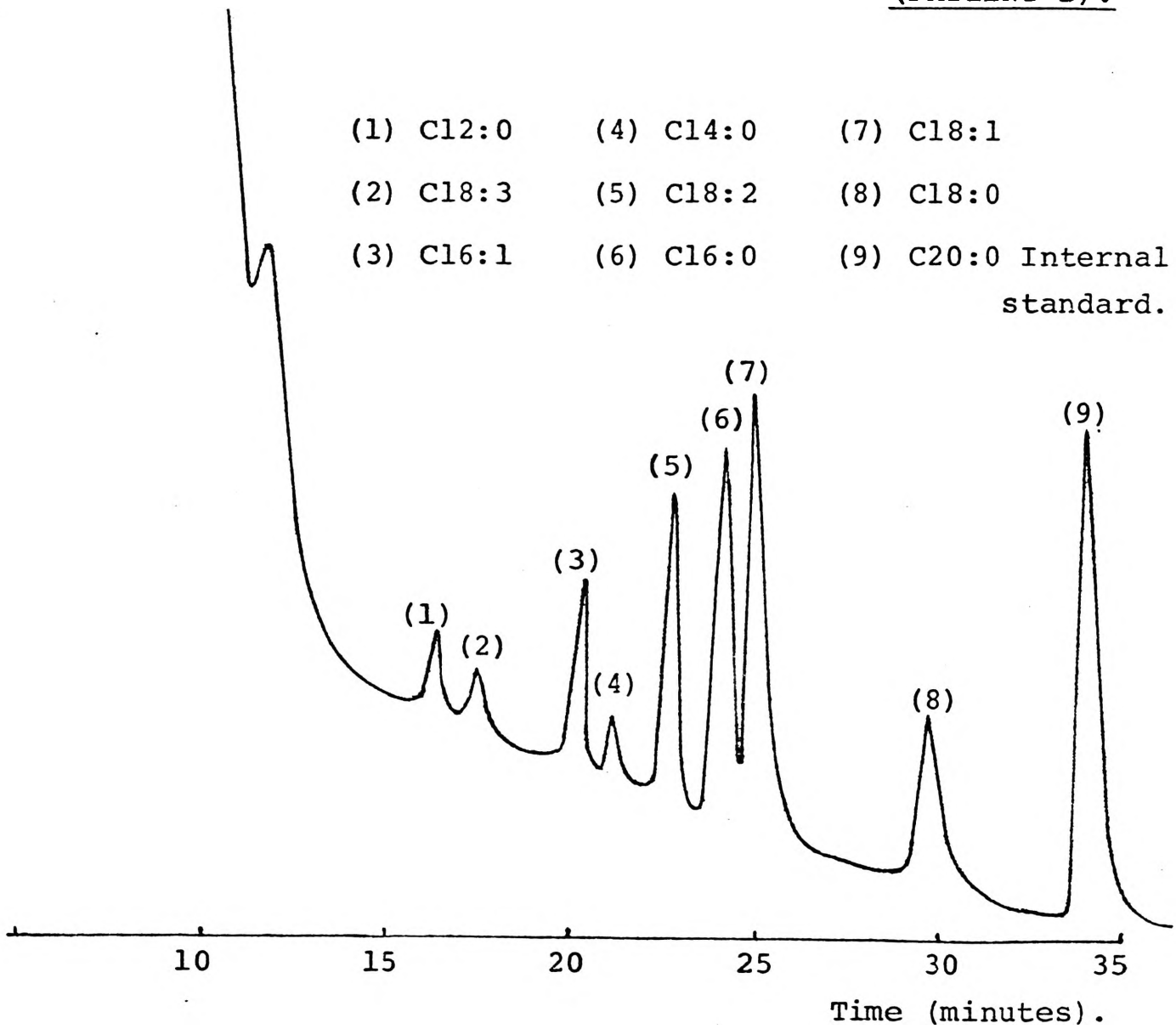
Ko and Royer[32] found that incomplete extraction of fatty acids from plasma occurred using this method, albeit a comparatively specific method. Percent recovery was shown to be dependent on the acid, ranging from 82.1% for C14:0 to 94.4% for C18:0. However, the addition of the internal standard prior to extraction should obviate this aspect.

The free fatty acid content of fresh blood (that of the author) was determined by the same method. Figure 17 depicts the chromatogram obtained. Once again the total free fatty acids (21.2 mg.%) was in good correlation with the expected level. Results are summarised in Table 17.

An addition (53.0 ugms.) of phytanic acid was added to the blood sample, as well as the internal standard. This was done to show that the peak corresponding to the 4-nitrobenzyl ester of the phytanic acid could be separated from the same esters of the common fatty acids, as occurred (Figure 17). As previously mentioned, people suffering from Refsum's Syndrome may have levels of phytanic acid as 30% of the total fatty acids in blood plasma. Consequently this method of analysis is suitable for determining phytanic acid levels in plasma or blood.

Another point is the correlation of the quantity of phytanic acid determined (53.5 ugms., Table 17) with the added quantity of the phytanic acid (53.0 ugms.), once again demonstrating the suitability of the method for quantitation.

FIGURE 18. CHROMATOGRAPHIC ANALYSIS OF FREE FATTY ACIDS, AS 4-NITROBENZYL ESTERS, EXTRACTED FROM DRIED HUMAN BLOOD SPOT (PATIENT B).



Instrument : Altex 312 Gradient.

Column : LiChrosorb C18 (25 cm.).

Eluent : Methanol/Water.

Gradient : Initial 80% Methanol. Final 100 % Methanol.
Exponent 0.3 . Time 30 minutes.
Hold 80% for 7 minutes, run to 87% then hold 5 minutes. Continue gradient.

Flow Rate : 1.5 mls./minute. Chart Speed : 0.5 cms./min.

Detector Sensitivity : 0.04 aufs. Injection Size : 20 ul.

TABLE 18

QUANTITATIVE ANALYSES OF FATTY ACIDS IN DRIED BLOOD SPOTS.

	Patient A			Patient B		
Sample Size	200 ul.			100 ul.		
Fatty Acid	Peak Area	Quantity Detmnd.	Percent Compsn.	Peak Area	Quantity Detmnd.	Percent Compsn.
C20:0 (Int. Std.)	60	5.0	-	235	10.0	-
C12:0	58	2.7	6.2	24	0.6	2.8
C14:0	29	1.5	3.4	19	0.5	2.4
C16:0	19.5	10.7	24.4	146	4.1	19.7
C16:1	39	3.2	7.3	59	2.5	12.0
C18:0	36	2.5	5.7	78	2.8	13.5
C18:1	193	14.1	32.2	175	6.5	31.3
C18:2	92	5.9	13.5	97	3.1	14.9
C18:3	54	3.2	7.3	24	0.7	3.4
Total fatty acids		43.8			20.8	
Total mg.%		21.9			20.8	

Comparison of the fatty acid compositions in the blood and plasma samples shows variations. However, as previously pointed out (page 3) fatty acid compositions are dependent on dietary factors.

C(ii). Quantitative Analyses of Free Fatty Acids in Dried Blood Spots.

The dried blood spots derived by applying aliquots of whole blood to filter paper are readily available from mass screening programmes. This blood spot technique may be substituted for more time consuming methods in many analytical schemes, and is ideal when specific blood tests are required at short notice from an infant who may not be immediately accessible.

The fatty acids in two microsamples of blood that had been treated in this fashion were extracted using the methanol/chloroform mixture[7] (2:1) then derivatised. Table 18 summarises the quantitation of the fatty acids, using arachidic acid (C20:0) as an internal standard, in two samples.

As expected, variations in the composition of the fatty acids were detected. However the total free fatty acids show good correlation with the normal level (20 mg.%).

Figure 18 represents the usual chromatogram obtained.

TABLE 19

	ACID LEVELS (mg.%)				
	EXPERIMENTAL				(a) LITERATURE
Fatty Acid	Plasma	Blood	Patient A	Patient B	
C12:0	0.4	0.3	1.4	0.6	0.1
C14:0	0.5	1.5	0.7	0.5	0.7
C16:0	4.6	4.0	5.4	4.1	3.9
C16:1	1.6	3.0	1.6	2.5	1.4
C18:0	1.0	2.0	1.2	2.8	0.8
C18:1	7.6	9.9	7.0	6.5	9.3
C18:2	2.1	0.1	3.0	3.1	2.3
C18:3	0.3	0.4	1.6	0.7	0.1
Total	18.1	21.2	21.9	20.8	

(a) Calculated from the figures in Table 1, Humans on a Random American Diet, assuming blood total free fatty acid level was 20 mg.% .

TABLE 20

REPRODUCIBILITY OF THE EXTRACTION, DERIVATISATION AND ANALYSIS BY HPLC.

Sample	Detector Sensitivity (aufs)	Quantity of C20:0 (ug.)	Peak Area Obtained (mm^2.)	Peak Area Adjusted (to 0.01 aufs)	Peak Area /Quantity (mm^2./ugm.)
Blood	0.04	66.5	432	1728	25.9
Plasma	0.04	66.5	60	120	24.0
Patient A	0.02	5.0	60	120	24.0
Patient B	0.01	10.0	235	235	23.5

Average Peak Area/Quantity of C20:0 = 24.6

Standard Deviation = 1.8

% Deviation = 7.3%

The reproducibility of the extraction, derivatisation and subsequent analysis by HPLC of fatty acids was determined for the arachidic acid internal standard.

The data summarised in Table 20 was obtained from the four blood analyses described. These were conducted over a period of several months, and showed a standard deviation of 7.3 percent.

C(iii). Comparison of Fatty Acid Levels in Analysed Samples.

As previously mentioned the total free fatty acids is reasonably constant in normal blood. However individual free fatty acid levels are dietary dependent. This may be the reason that literature values of absolute levels of all the common fatty acids were not found.

A compilation of results of all samples analysed is given in Table 19. These are compared with the figures in Table 1, Humans on a Random American Diet, assuming the total free fatty acids in blood was 20 mg.%. This is an extrapolation as Table 1 figures were determined as fatty acid residues of triglycerides in the adipose tissue.

The observed correlation with experimental figures would indicate the proposed source of free fatty acids in the blood stream, namely lipolysis of triglycerides in adipose tissue,[5] is reasonable.

A P P E N D I X

Within the linear response of the detector, the ratio of the peak area per unit weight of ester on the column is a constant (equal to the slope of the calibration curve).

The weight of ester introduced to the column is directly proportional to the total ester weight in the sample solution formed after pyrolysis as the injected volume of solution is a fraction of the total solution volume.

$$\text{Thus} \quad \frac{\text{Peak Area}}{\text{Wt. ester in solution}} = \text{constant} \qquad \text{(a)}$$

As the formation of ester by pyrolysis of the acid salt is complete,

$$\text{Wt. of ester in solution} = (\text{Wt. of acid extracted (from sample)}) \times \frac{\text{M. Wt. ester}}{\text{M. Wt. acid.}}$$

Substituting into (a),

$$\frac{\text{Peak Area}}{\text{Wt. acid in sample}} \times \frac{\text{M. Wt. acid}}{\text{M. Wt. ester}} = \text{constant} \qquad \text{(b)}$$

Using an internal standard, for any UV absorbing species,

$$\frac{\text{Peak Area per unit wt. of species to be analysed}}{\text{Peak Area per unit wt. of internal standard}} = \text{constant.}$$

Substituting (b),

$$\frac{\left(\frac{\text{Peak Area for acid of interest}}{\text{Wt. of acid in sample}}\right) \times \frac{\text{M.Wt. acid}}{\text{M.Wt. ester}}}{\left(\frac{\text{Peak Area for C20:0}}{\text{Wt. C20:0 added}}\right) \times \frac{\text{M.Wt. C20:0}}{\text{M.Wt. C20:0 ester}}} = \text{constant.}$$

Thus for any given acid in two samples (1) and (2)

$$\frac{\left(\frac{\text{Peak Area for acid of interest (1)}}{\text{Wt. acid in sample (1)}}\right) \times \frac{\text{M.Wt. acid}}{\text{M.Wt. ester}}}{\left(\frac{\text{Peak Area for C20:0 (1)}}{\text{Wt. C20:0 added to sample (1)}}\right) \times \frac{\text{M.Wt. C20:0}}{\text{M.Wt. C20:0 ester}}}$$

$$= \frac{\left(\frac{\text{Peak Area for acid of interest (2)}}{\text{Wt. acid in sample (2)}}\right) \times \frac{\text{M.Wt. acid}}{\text{M.Wt. ester}}}{\left(\frac{\text{Peak Area for C20:0 (2)}}{\text{Wt. C20:0 added to sample (2)}}\right) \times \frac{\text{M.Wt. C20:0}}{\text{M.Wt. C20:0 ester}}}$$

Cancelling the Molecular Weight terms,

$$\frac{\left(\frac{\text{Peak Area for acid of interest (1)}}{\text{Wt. acid in sample (1)}}\right)}{\left(\frac{\text{Peak Area for C20:0 (1)}}{\text{Wt. C20:0 added to sample (1)}}\right)} = \frac{\left(\frac{\text{Peak Area for acid of interest (2)}}{\text{Wt. acid in sample (2)}}\right)}{\left(\frac{\text{Peak Area for C20:0 (2)}}{\text{Wt. C20:0 added to sample (2)}}\right)}$$

But the columns denoted "Constant" in Tables 12, 13 and 14 are given by :

$$\text{"Constant"} = \frac{\left(\frac{\text{Peak Area for acid of interest (measured)}}{\text{Wt. acid in sample (known)}}\right)}{\left(\frac{\text{Peak Area for C20:0 (measured)}}{\text{Wt. C20:0 added to sample (known)}}\right)}$$

This constant was then computed.

Thus, the unknown weight of an acid in a sample may be determined using C20:0 as an internal standard, from the chromatogram of the resulting solution after 4-nitrobenzylating by :

$$\frac{\dfrac{(\text{Peak Area of acid})}{(\text{Wt. acid in sample})}}{\left(\dfrac{\text{Peak Area of C20:0}}{(\text{Wt. C20:0 added to sample})}\right)} = \text{Constant (from Table 14)}$$

Rearranging,

$$\text{Wt. of acid in sample} = \frac{(\text{Peak Area for acid}) \times (\text{Wt. C20:0 added})}{(\text{Peak Area for C20:0}) \times (\text{Constant}_{\text{Table 14}})}$$

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